Universidade de Aveiro Departamento de Biologia
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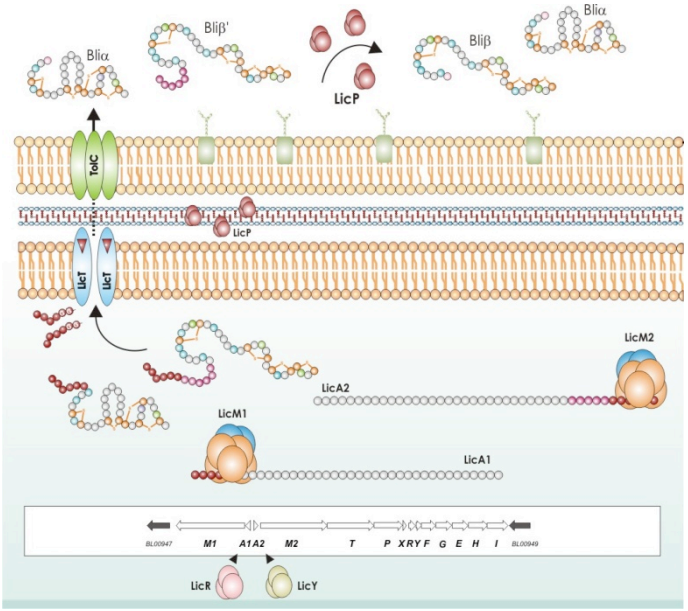
Tânia Isabel Sousa Caetano

Biossíntese da lichenicidina e pesquisa de novos péptidos antibacterianos

Lichenicidin biosynthesis and search for novel antibacterial peptides



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Lichenicidin biosynthesis and search for novel antibacterial peptides

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica da Doutora Sónia Mendo, Professor auxiliar do Departamento de Biologia da Universidade de Aveiro e da Doutora Ana Domingos, investigadora da Unidade de Tecnologias de Proteínas e Anticorpos Monoclonais do Instituto de Higiene e Medicina Tropical. A coordenação empresarial foi realizada pela licenciada Júlia Oliveira, gerente da propriedade intelectual dos Laboratórios Medinfar, SA.

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Especialmente para os meus adorados pais, Adelina e Fernando e para o meu irmão João Paulo.

o júri

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palavras-chave

B. licheniformis, bacteriocinas, lantibióticos, lichenicidina, expressão heteróloga, péptidos não ribossomais, péptidos antibacterianos, dissociação morfológica

resumo

A estirpe *Bacillus licheniformis* I89 possui a capacidade de produzir alguns compostos com actividade antibacteriana. No presente estudo, a separação desses compostos foi realizada através da aplicação de vários procedimentos, incluindo extracção em fase sólida e cromatografia liquida de alta pressão. Dois destes compostos bioactivos constituem o lantibiótico de classe II lichenicidina e são caracterizados pela massas molecular de 3250 Da (Bliα) e 3020 Da (Bliβ). O cluster responsável pela biossíntese da lichenicidina foi heterologamente expresso em *Escherichia coli*, constituindo a primeira descrição da produção de um lantibiótico totalmente *in vivo* num hospedeiro Gram-negativo. Este sistema foi subsequentemente explorado com o objectivo de relacionar cada proteína codificada no cluster genético da lichenicidina na produção dos péptidos Bliα e Bliβ. O desenvolvimento do sistema de *trans* complementação possibilitou a produção de variantes destes péptidos. A análise das massas moleculares destas variantes assim como a análise dos padrões de fragmentação obtidos por MS/MS permitiu a revisão de algumas das características estruturais previamente proposta para Bliα e Bliβ. A análise dos genes hipoteticamente envolvidos na protecção da estirpe produtora contra a acção antibiótica da lichenicidina revelou, que em *E. coli*, a sua ausência não resulta no aumento da susceptibilidade a este composto. Verificou-se também que a presença destes genes não é essencial para a produção de lichenicidina em *E. coli*. Foi também confirmado experimentalmente que a membrana externa da *E. coli* constitui uma barreira natural para a entrada dos péptidos na célula. De facto, uma das características intrigantes da produção de lichenicidina por uma bactéria de Gram negativo reside no mecanismo de transporte dos dois péptidos através da membrana externa. Neste estudo foi demonstrado que na ausência da proteína de membrana TolC, a massa molecular de Bliα e Bliβ não foi identificada no sobrenadante de *E. coli*, demonstrando assim que a sua presença no ambiente extra-celular não se devia a um processo de lise bacteriana. Foi ainda avaliada a capacidade da maquinaria biossintética da lichenicidina para produzir o lantibiótico haloduracina, através do processamento de chimeras lichenicidina-haloduracina, contudo, os resultados foram negativos. Verificou-se ainda que em determinadas condições de incubação, a diferenciação da morfologia original da estirpe *B. licheniformis* I89 pode ocorrer. Esta dissociação implicou a transição da colónia parental e rugosa para uma colónia de aparência mais simples e suave. Desta forma, as diferenças das duas morfologias em termos de taxa de crescimento, esporulação e actividade antibiótica foram investigadas. Considerando especificamente Bliα e Bliβ verificou-se que a abundância destes péptidos nas culturas do fenótipo fino é geralmente inferior aquela identificada nas culturas do fenótipo parental. Por último, a diversidade de elementos genéticos constituintes de péptido sintetases não ribossomais (NRPS) foi investigada em lagoas no centro de Portugal e em solos provenientes de caves do sul de Portugal, revelando a presença de potenciais novas NRPS nestes ambientes.

keywords

B. licheniformis, bacteriocins, lantibiotics, lichenicidin, heterologous expression, nonribosomal peptides, antibacterial peptides, morphological dissociation

abstract

Bacillus licheniformis I89 has the ability to produce some antibacterial compounds. In the present study, the separation of such compounds was achieved by the application of several procedures, including solid phase extraction and preparative high-pressure liquid chromatography. Two of these compounds constitute the class II lantibiotic lichenicidin and are characterized by the molecular masses of 3250 Da (Bliα) and 3020 Da (Bliβ). The lichenicidin gene cluster was successfully expressed in *Escherichia coli*, constituting the first report of a complete lantibiotic gene cluster heterologous expression in a Gram-negative host. This system was further exploited to characterize and assign the function of the proteins encoded in the biosynthetic gene cluster. Moreover, a *trans* complementation system was developed for the expression of Bliα and Bliβ mutants *in vivo*. The assignment of essential amino acid residues for bioactivity was investigated by generation of Ala-mutants. Also, several features of Bliα and Bliβ structures were revised by analysis of MS/MS fragmentation patterns obtained for wild type and mutated peptides. Regarding the lichenicidin self-protection in *E. coli*, it was found that immunity genetic determinants are not essential to Bliα and Bliβ production. Furthermore, it was experimentally confirmed that the *E. coli* outer membrane constitutes a natural barrier to the biological activity of lichenicidin. An intriguing feature of the lichenicidin production by *E. coli* lies in the export mechanism of Bliα and Bliβ peptides. Herein, it was demonstrated that the presence of these peptides in the *E. coli* supernatants resulted from their translocation through the bacterial cell wall. In fact, it was found that in the absence of the outer membrane protein TolC none of the lichenicidin peptides could be detected in the bacterial supernatants. The potential of the *E. coli* lichenicidin expression system to produce the closely related lantibiotic haloduracin through the biosynthetic processing of lichenicidin-haloduracin chimeras was also attempted in the present study, however, without success.

It was found that under certain circumstances of incubation, *B. licheniformis* I89was able to differentiate from its parental rough phenotype to a more simple and smooth morphology. The differences in terms of growth, sporulation and antagonistic activity of both phenotypes were investigated in two different culture media. Regarding the production of lichenicidin peptides, it was found that the abundance of Bliα and Bliβ in extracts from the smooth phenotype cultivated in TSB was lower than that detected in the rough phenotype extracts. Finally, the screening of genetic elements associated with nonribosomal peptide synthetases was performed in Portuguese lagoons and soil from caves using a culture-independent approach. A wide variety of adenylation domains was identified, providing evidence that potentially novel NRPSs are present in these environments.

"Posso ter defeitos, viver ansioso e ficar irritado algumas vezes,
Mas não esqueço de que minha vida
É a maior empresa do mundo…
E que posso evitar que ela vá à falência.
Ser feliz é reconhecer que vale a pena viver
Apesar de todos os desafios, incompreensões e períodos de crise.
Ser feliz é deixar de ser vítima dos problemas e
Se tornar um autor da própria história…
É atravessar desertos fora de si, mas ser capaz de encontrar
Um oásis no recôndito da sua alma…
É agradecer a Deus a cada manhã pelo milagre da vida.
Ser feliz é não ter medo dos próprios sentimentos.
É saber falar de si mesmo.
É ter coragem para ouvir um "Não"!!!
É ter segurança para receber uma crítica,
Mesmo que injusta…

Pedras no caminho?
Guardo todas, um dia vou construir um castelo…"

Fernando Pessoa

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List of Abbreviations

A domain	Adenylation domain
ACN	Acetonitrile
Amp	Ampicillin
Apra	Apramycin
AR	Algar Romão
AU	Arbitrary units
CFU	Colony forming unit
Clo	Chloramphenicol
dH_2O	Distilled water
dNTP's	Deoxynucleotide triphosphates
Dha	2,3-didehydroalanine
Dhb	2,3-didehydrobutyrine
DNA	Deoxyribonucleic acid
eDNA	Environmental deoxyribonucleic acid
Elk	Epilancin K7
Epi	Epidermin
Hal	Haloduracin
HPLC	High-pressure liquid chromatography
HR-ESI-MS	High resolution electrospray mass spectra
Kan	Kanamycin
Lan	Lanthionine
Las	Lactocin S
LB	Lake Barrinha de Mira
LC-ESI-MS	Liquid chromatography–electrospray ionization mass spectrometry
Lct	Lacticin 481
Lic	Lichenicidin
LM	Lake Mira
Ltn	Lacticin 3147

LV	Lake Vela
MC	Minimal inhibitory concentration
MCP	Micro-channel plate
MeLan	Methyllanthionine
MFP	Membrane fusion protein
Mrs	Mersacidin
MRSA	Methicillin-resistant *Staphylococcus aureus*
MS	Mass spectrometry
Mut	Mutacin
Nis	Nisin
NMR	Nuclear magnetic resonance
NRPS	Nonribosomal peptide synthetase
Nuk	Nukacin ISK-1
Obu	2-oxobutyryl
OM	Outer membrane
ORF	Open reading frame
PCR	Polymerase chain reaction
Pep	Pep5
Pne	Pneumococcin
rep-PCR	Repetitive extragenic palindromic-PCR
RFLP	Restriction fragment length polymorphism
rRNA	Ribosomal ribonucleic acid
RT	Room temperature
R_t	Retention time
SIVB	Semi-*in vitro* biosynthesis
Spa	Subtilin
SPE	Solid phase extraction
TOF	Time of flight
US	United States of America
UV	Ultra-violet
VRE	Vancomycin-resistant *Enterococcus*
VT	Vale Telhado

Structure of this dissertation

The main objective of the present dissertation was to characterize the antibacterial compounds produced by the *B. licheniformis* I89 strain, considering their biosynthetic pathway, structure and biological activity. Also, a screening of some environments was performed to identify the presence of genetic determinants associated with the biosynthetic pathway of conventional antibiotics.

Thus, this dissertation is composed by a general introduction (chapter I), incorporating the state-of-art in the field of antibacterial class I bacteriocins as well as a general overview of the traditional antibiotics biosynthesis. In the following chapters (chapter II-VII) are presented and discussed the results obtained in the studies performed. Each of these chapters includes a brief introduction, the results and discussion of the major findings and the experimental procedures. Finally, a unifying overview and discussion of all the themes addressed along this dissertation is presented in the final chapter (chapter VIII).

CHAPTER I

General Introduction

More than one century ago, it was recognized for the first time that bacteria have the intriguing ability to inhibit the growth of other bacteria as part of an antibiosis process. Several years later, Sir Alexander Fleming also noticed that the mold *Penicillium notatum* had the ability to kill the bacteria *Staphylococcus aureus* and *Haemophilus influenzae* and related such phenomenon with the production of a compound designated by himself as penicillin (1929). However the importance of his findings was only recognized in the late 1930's and early 1940's by Florey and Chain, who would finally prompt the industrial production and clinical application of penicillin. The term "antibiotic" to compounds such as penicillin was attributed by the soil microbiologist Selman Waksman, who defined it as any substance produced by a microorganism that is antagonistic to the growth of other microorganisms in high dilutions (Waksman, 1947; Levy, 2002). Selman Waksman and his student Schatz also isolated the antibiotic streptomycin, that was produced by the soil bacteria *Streptomyces griseus* and was able to control the growth of the Gram-negative *Mycobacterium tuberculosis*, being the first therapeutic agent to offer hope to patients with tuberculosis (Jones *et al.*, 1944). Subsequent studies resulted in the discovery of novel different antibiotics including gramicidin, chloramphenicol, tetracycline, polymyxin and neomycin.

Research developments of antibacterial compounds also implied the investigation of their biosynthetic pathways. Considering this aspect it was found that bacteria have the ability to produce two distinct classes of antimicrobial compounds: those that are ribosomally synthesized, which are designated by bacteriocins and those that are non-ribosomally produced by multi-enzymatic complexes called nonribosomal peptide synthetases (NRPSs) (Nes *et al.*, 2007). The main focus of this thesis relies on aspects related with the biosynthesis of a class of bacteriocins – the lantibiotics.

1.1 Bacteriocins - a general overview

Bacteriocins are a highly diverse group of ribosomally compounds produced by a bacteria that are active against other bacteria, either from the same species (narrow spectrum) or across a genera (broad spectrum) (Cotter *et al.*, 2005a). The first reported bacteriocin was colicin V, which was identified by Andre Gratia in 1925. Colicin V is a heat-labile peptide present in cultures of *Escherichia coli* V with activity against *E. coli* ϕ (Gratia, 1925). In 1928, another bacteriocin was also described by Rogers (1928) although it was only characterized in 1971, as nisin A (Gross and Morell, 1971). Nisin A is produced by *Lactococcus lactis*, a widely used bacterium in cheese production. Almost two decades later, Klaenhammer (1988) predicted that 99 % of all bacteria should have the ability to synthesize at least one bacteriocin (Maqueda *et al.*, 2008). Nowadays and according with the bacteriocins database BACTIBASE (Hammami *et al.*, 2007), at least 177 sequences of bacteriocins are described, of which 88 % are synthesized by Gram-positive bacteria, 10 % by Gram-negative bacteria and approximately 2 % by members of the Archae domain. A total of 31 genera were identified as producers, with the lactic acid bacteria (LAB) representing the most predominant group of producers (64 %), followed by *Bacillus* (8.5 %), *Escherichia* (6.2 %), *Staphylococcus* (4.5 %) and *Streptomyces* (2.8 %).

The bacteriocins produced by Gram-positive are generally smaller than 6 KDa, whereas those produced by Gram-negative are often proteins larger than 20 KDa, being colicin V and microcins exceptions (smaller than 10 KDa) (Nissen-Meyer and Nes, 1997). Moreover, the Gram-positive antimicrobial peptides are generally non-toxic to eukaryotic cells and have a much broader inhibitory spectra when compared to those of Gram-negative bacteria (Nes *et al.*, 2007).

The establishment of a proper classification scheme for these compounds has been difficult since some authors defend that the available systematics lacks consistency and coherence (van Belkum and Stiles, 2000; Zouhir *et al.*, 2010). Initially, these compounds were classified into two main classes: the class I, consisting of the modified bacteriocins lantibiotics and the class II consisting of the unmodified peptide bacteriocins (Nissen-Meyer and Nes, 1997). Presently, the bacteriocins are grouped into different classes based on different characteristics such as the producer microorganisms, molecular weight, specific antibacterial activity, physical properties, chemical structures and mode of action (de Jong *et al.*, 2006; Nes *et al.*, 2007). According to this scheme, it has been proposed to divide the bacteriocins into five classes (de Jong *et al.*, 2006):

Class I: post-translationally modified bacteriocins, designated as lantibiotics

Class II: non-modified heat stable bacteriocins

Class III: non-modified large heat-labile bacteriocins

Class IV: non-modified circular bacteriocins

Class V: non-modified complex bacteriocins carrying lipid or carbohydrate moieties

In the following sections only the lantibiotics class of bacteriocins will be discussed, since these compounds are the main focus of the present thesis.

1.2 Lantibiotics – class I bacteriocins

Lantibiotics constitute the first class of bacteriocins and are described as ribosomally synthesized peptides, which undergo extensive post-translational modification to form their biologically active forms (de Vos *et al.*, 1995; Sahl *et al.*, 1995; Sahl and Bierbaum, 1998). Contrary to other classes of bacteriocins, lantibiotics are exclusively produced by Gram-positive bacteria. Although not recognized at the time, the first report of a lantibiotic goes back to 1928, the same year of penicillin discovery, when Rogers observed that metabolites from *Lactococcus lactis* (LAB bacteria) could inhibit other LAB bacteria (Rogers, 1928). In 1944, Mattick and Hirsch (1944) partially purified and analyzed the antagonistic compound responsible for such phenomenon and later, Gross and Morell (1971) proposed a primary structure for the peptide that became known as nisin A. Nevertheless, the term lantibiotic was only introduced a few years later by Schnell *et al.* (1988) to describe the epidermin peptide produced by *Staphylococcus epidermidis* as a lanthionine-containing antibiotic. The characteristic lanthionine (Lan) amino acids were isolated for the first time by Horn and coworkers (1941) from wool (*lana* in latin) and consist of two alanines cross-linked via a thioether linkage that connects their β-carbons. As indicated by their designation, the presence of Lan and usually methyllanthionine (MeLan) aminoacids is the unifying structural motif of all the lantibiotics (Chatterjee *et al.*, 2005). In the beginning of the 70's it was proposed that the Lan and MeLan rings were generated by intramolecular additions of cysteines to the α, β-unsaturated amino acids 2,3-didehydroalanine (Dha) and (Z)-2,3-didehydrobutyrine (Dhb), respectively (Figure 1). Also, it was hypothesized that these Dha and Dhb amino acids, which were frequently found in lantibiotics, originated from the dehydration of serine or threonine residues, respectively (Ingram, 1969; Ingram, 1970). These hypotheses were only confirmed in the late 80's, beginning of the 90's when the first biosynthetic gene clusters involved in the lantibiotics production were sequenced (Chatterjee *et al.*, 2005). These clusters generally encode all the proteins involved in the production of the bioactive compound, including i) the synthesis of the prepropeptide, ii) dehydration and cross-linkage reactions, iii) cleavage of the leader sequence, iv) secretion and v) self-protection mechanisms (McAuliffe *et al.*, 2001b).

Lantibiotics have been considered intriguing molecules because of their unique characteristics such as their biochemistry, genetic regulation and biological activities (Willey and van der Donk, 2007). Their bactericidal activity, at nanomolar concentrations, against a variety of Gram-positive bacteria, including multiresistant strains, has been the most promising and explored of their potential applications. However, not all of these compounds harbor this ability.

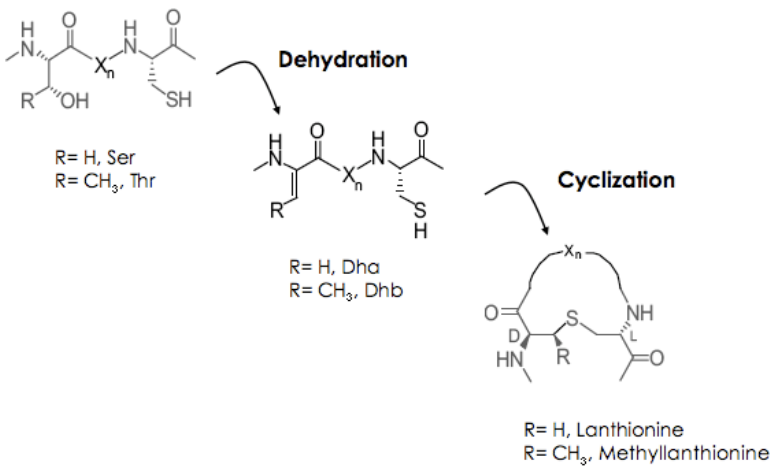


Figure 1: Formation of lanthionine and methyllanthionine rings. Adapted from Willey and van der Donk (2007)

1.2.1 Classification schemes

Two main classification schemes for lantibiotics have been proposed: the first and the oldest is based on the topology of the lantibiotics ring structures and their biological activities (Jung and Sahl, 1991) while the second is based on the peptides biosynthetic pathway and on their mechanism of action (Pag and Sahl, 2002; Willey and van der Donk, 2007). According to the first system (Jung and Sahl, 1991), the lantibiotics were divided into two distinct groups: i) the type-A lantibiotics possessing an elongated flexible configuration, which are able to disturb the bacterial membrane due to the presence of a positive net charge and ii) the type-B lantibiotics characterized by a globular and compact structure (usually without a net charge), which biological activity involves the inhibition of bacterial enzymes (Figure 7). However, with the successive discovery of new peptides, the type A lantibiotics were further sub-grouped into type-A(I) and type-A(II) (de Vos *et al.*, 1995). The type-A(II) lantibiotics comprise peptides characterized by a linear N-terminus and a globular C-terminus, whereas the type-A(I) are more elongated lantibiotics (Figure 7). The prototypes of each class are based on those peptides with experimentally

established structures, such as the type-A(I) nisin, epidermin and pep5; the type-A(II) lacticin 481 and the type-B mersacidin and cinnamycin (Bierbaum and Sahl, 2009) (Figure 7 and Table 1).

Table 1: Overview of lantibiotics grouped according with de Vos *et al.* (1995) classification and their similarities to peptides with established structures. The producer strains and the lantibiotics molecular weights are also included. Adapted from Chatterjee *et al.* (2005) and Bierbaum and Sahl (2009).

	Lantibiotic	Producer strain	MW (Da)	Class	Reference
Type-A (I)	**Nisin group**				
	Nisin A	*Lactococcus lactis* ATCC 11454	3353	I	(Gross and Morell, 1971)
	Nisin Z	*Lactococcus lactis* N8	3330	I	(Mulders *et al.*, 1991)
	Subtilin	*Bacillus subtilis* ATCC 6633	3317	I	(Gross *et al.*, 1973)
	Ericin S	*Bacillus subtilis* A1/3	3442	I	(Stein *et al.*, 2002)
	Ericin A	*Bacillus subtilis* A1/3	2986	I	(Stein *et al.*, 2002)
	Microbisporicins	*Microsbiospora sp.*	2246/2230	I	(Castiglione *et al.*, 2008)
	Epidermin group				
	Epidermin	*Staphylococcus epidermis* Tu3298	2164	I	(Allgaier *et al.*, 1986)
	Gallidermin	*Staphylococcus gallinarium* Tu3928	2164	I	(Kellner *et al.*, 1988)
	Mutacin III	*Streptococcus mutans* UA787	2266	I	(Qi *et al.*, 1999b)
	Mutacin I	*Streptococcus mutans* CH43	2364	I	(Qi *et al.*, 2000)
	Pep5 group				
	Pep5	*Staphylococcus epidermidis* 5	3488	I	(Kellner *et al.*, 1989)
	Epilancin K7	*Staphylococcus epidermidis*	3032	I	(van de Kamp *et al.*, 1995)
Type-A (II)	**Lacticin 481 group**				
	Lacticin 481	*Lactococcus lactis* CNRZ 481	2901	II	(Piard *et al.*, 1992)
	Lactocin S	*Lactococcus sakei* L45	3764	II	(Skaugen *et al.*, 1997)
	Mutacin II	*Streptococcus mutans* T8	3245	II	(Novak *et al.*, 1994)
	Nukacin ISK-1	*Staphylococcus warneri* ISK-1	2960	II	(Sashihara *et al.*, 2000)
	Plantaricin C	*Lactococcus plantarum* LL441	2880	II	(Turner *et al.*, 1999)
	Ruminococcin A	*Ruminococcus gnavus*	2675	II	(Dabard *et al.*, 2001)
	Salivaricin A	*Streptococcus salivarius* 20P3	2315	II	(Ross *et al.*, 1993)
	Variacin	*Micrococcus varians* MCV8	2658	II	(Pridmore *et al.*, 1996)
Type B	**Cinnamycin group**				
	Cinnamycin	*Streptomyces cinnamoneus*	2042	II	(Fredenhagen *et al.*, 1990)
	Duramycin	*Streptoverticillium hachijoense* DSM 40114	2014	II	(Fredenhagen *et al.*, 1990)
	Ancovenin	*Streptomyces* sp.	1959	II	(Kido *et al.*, 1983)
	Mersacidin group				
	Actagardine	*Actinoplanes linguriae* ATCC 31048	1890	II	(Zimmermann and Jung, 1997)
	Mersacidin	*Bacillus* sp. HIL Y-85,54728	1825	II	(Chatterjee *et al.*, 1992)
	Michiganin A	*Clavibacter michiganensis*	2145	II	(Holtsmark *et al.*, 2006)
Two-component			α β		
	BHT	*Streptococcus rattus* BHT	3375 2802	II	(Hyink *et al.*, 2005)
	Cytolysin L*	*Enterococcus faecalis*	4164 2631	II	(Gilmore *et al.*, 1994)
	Haloduracin	*Bacillus halodurans* C-125	2332 3099	II	(McClerren *et al.*, 2006)
	Lacticin 3147	*Lactococcus lactis* DPC3147	3322 2847	II	(Martin *et al.*, 2004)
	Lichenicidin	*Bacillus licheniformis*	3250 3020	II	(Begley *et al.*, 2009)
	Plantaricin W	*Lactobacillus plantarum* LMG 2379	3223 3099	II	(Holo *et al.*, 2001)
	Staphylococcin C55	*Staphylococcus aureus* C55	3339 2993	II	(Navaratna *et al.*, 1999)
	Smb	*Streptococcus mutans* GS5	nd	II	(Yonezawa and Kuramitsu, 2005)
Others	**Non-antimicrobial peptides/Lantipeptides**				
	SapB	*Streptomyces coelicolor*	2026	III	(Kodani *et al.*, 2004)
	SapT	*Streptomyces tendae*	2032	III	(Kodani *et al.*, 2005)
	AmfS	*Streptomyces griseus*	nd	III	(Ueda *et al.*, 2002)
	Labyrinthopeptin A2	*Actinomadura namibiensis* DSM 6313	1065	III	(Meindl *et al.*, 2010)

As abovementioned, not all the lantibiotics function strictly as antimicrobial agents. For instance, cinnamycin and the related duramycins are potent inhibitors of phospholipase A2 (Fredenhagen *et al.*, 1990). Also, duramycin increases chloride secretion in the lung epithelium and has been evaluated for the mucus clearing secretions from the lungs associated with cystic fibrosis diseases (Willey and van der Donk, 2007). Cytolysin, apart from the inhibition of Gram-positive bacteria, also functions as a virulence factor, promoting the lysis of erythrocytes and polymorphonuclear leukocytes (Cox *et al.*, 2005). The SapB and SapT compounds do not exhibit antimicrobial activity and are thought to function as biosurfactants, releasing the surface tension at the colony-air interface to facilitate the emergence of nascent aerial hyphae of *Streptomyces* spp. strains (Tillotson *et al.*, 1998; Kodani *et al.*, 2004; Kodani *et al.*, 2005). Regarding the recently described labyrinthopeptins, bioactivity assays of labyrinthopeptin A2 revealed that it has an excellent efficacy against neuropathic pain in an *in vivo* mouse model (Meindl *et al.*, 2010).

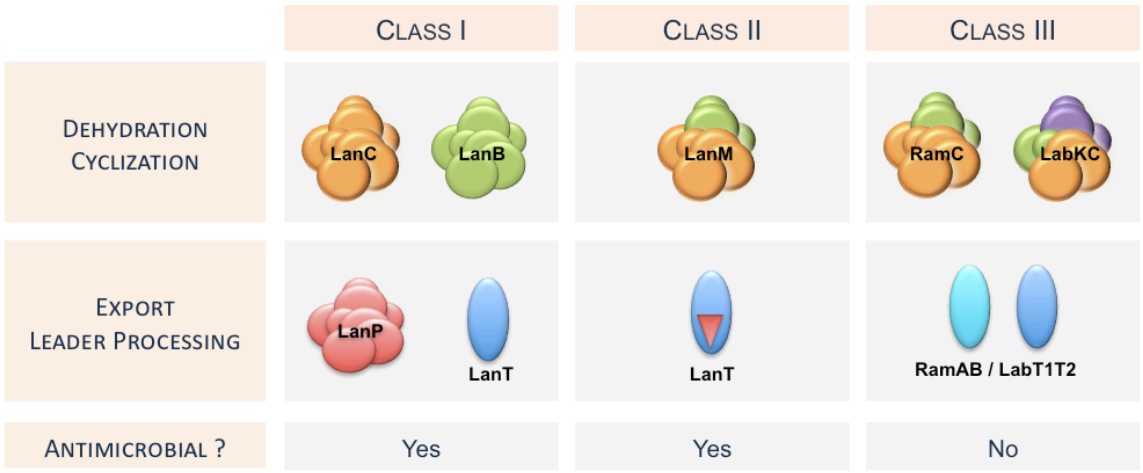


Figure 2: Classification of lantibiotics according with the parameters established by Pag and Sahl (2002) and Willey and van der Donk (2007).

1.2.2 General overview of lantibiotics biosynthesis

The lantibiotic biosynthetic gene clusters are often present on the chromosome of the producer strain, however they can also be found on conjugative transposable elements (e.g. nisin) and plasmids (e.g. lacticin 3147) (Horn *et al.*, 1991; Ryan *et al.*, 1996). There is evidence that at least some parts of these gene clusters are organized in operons, many

of them consisting of several transcriptional units. Also, a weak terminator structure is often found in between the structural gene and its downstream ORF (McAuliffe *et al.*, 2001b). Historically, the genes that constitute each lantibiotic biosynthetic cluster are generally describe with the suffix *lan* (de Vos *et al.*, 1995). When a particularization is necessary, this suffix is substituted by the specific designation of the lantibiotic in question.

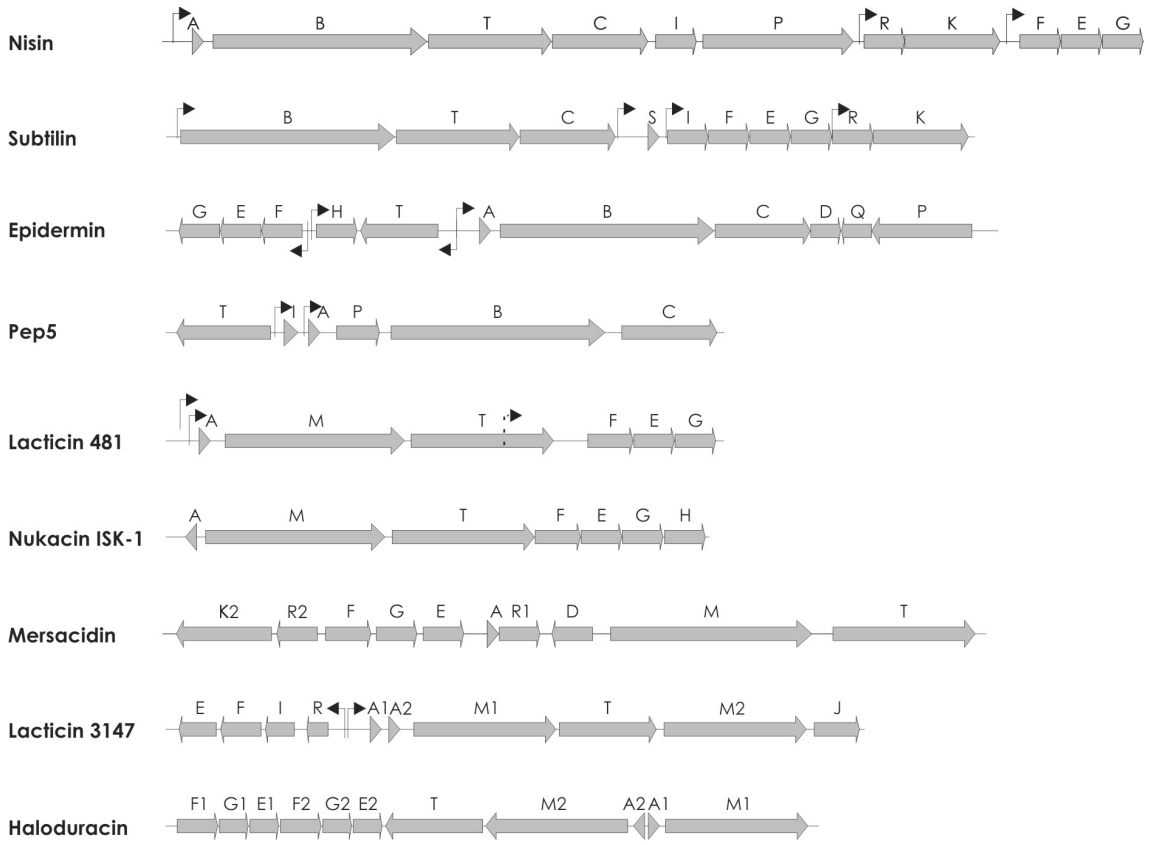


Figure 3: Representation of the biosynthetic gene clusters involved in the production of the most representative lantibiotics from each class. Transcriptional units, when known, are indicated by black arrows. Dashed arrow represents a putative promoter. The subtilin structural gene corresponds to *lanS*. Adapted from Willey and van der Donk (2007).

Although the gene order, complexity, and transcriptional organization of the various clusters can be different, some essential genes are always present (Figure 3) (Asaduzzaman and Sonomoto, 2009). These include the structural gene (*lanA*) encoding the precursor prepropeptide, which will be the target for post-translational modifications. For historical reasons the structural genes of subtilin and cytolysin do not follow this rule, being refereed as *spaS* and *cylL*$_L$/*cylL*$_S$, respectively. The post-translational modification of the LanA C-terminus is essential for the production of the bioactive lantibiotic. Accordingly, the *lanB* and *lanC* genes found in class I lantibiotic clusters encode the LanB and LanC

proteins responsible for the dehydration of Ser and Thr residues and the formation of the thioether rings, respectively (Figure 3 and Figure 4). In class II lantibiotics, a single bifunctional LanM protein encoded by *lanM* catalyzes these two reactions (Figure 3 and Figure 4). After the intervention of these enzymes, the leader sequences of the modified LanA peptides are removed during or after its translocation to the bacterial environment. In the biosynthesis of class I lantibiotics this step is carried out by two distinct enzymes, LanP and LanT, whereas in class II lantibiotics a single LanT protein combines both activities (Figure 3 and Figure 4) (Chatterjee *et al.*, 2005; Willey and van der Donk, 2007).

Additionally, some biosynthetic clusters possess two-component sensory system genes (e.g. *lanK* and *lanR*) encoding proteins involved in the regulation of the lantibiotic synthesis. Several gene clusters also contain a second transport system comprising three genes *lanE*, *lanF* and *lanG* that have been associated with the self-protection of the producer strain. Other genes associated with specific post-translational modifications can also be identified in lantibiotics' gene clusters. Examples include the genes encoding a hydroxylase (*ltnJ* and *cinX*) that oxidizes an asparagine residue in β-position of some lantibiotics, such as lacticin 3147 and cinnamycin (Figure 3) (Willey and van der Donk, 2007).

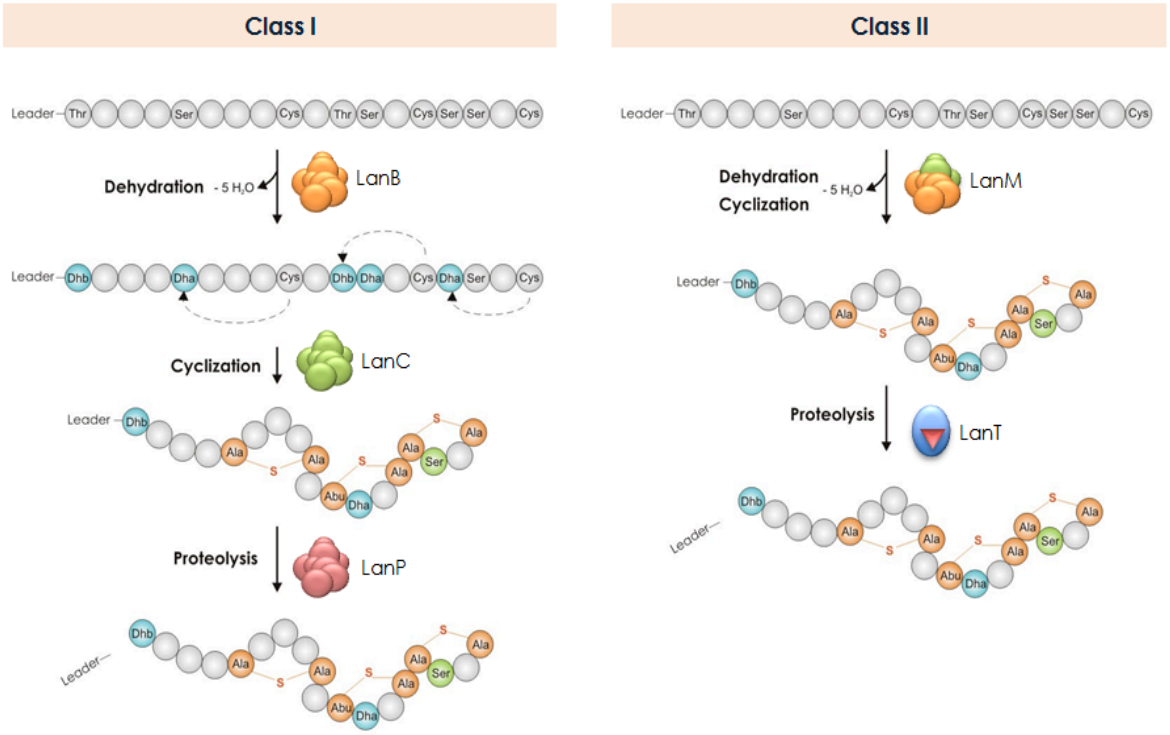


Figure 4: General overview and comparison of the dehydration, cyclization and leader sequence removal steps involved in the biosynthesis of class I and class II lantibiotics.

The biosynthesis of two-component lantibiotics such as lacticin 3147 and haloduracin involves the action of the classical class II enzymes. The production of these peptides requires the expression of two structural genes, the *lanA1* and *lanA2* (Figure 3). Subsequently, LanA1 and LanA2 prepropeptides are modified by the distinct LanM1 and LanM2 enzymes, respectively. However, the export and removal of the leader sequence of LanA1 and LanA2 is directed by the same LanT protein. Some of these lantibiotics (e.g. Halβ from haloduracin and Plwβ from plantaricin W) require a second proteolysis step before they become completely bioactive. The protease responsible for this reaction is specific for one of the two peptides and it is not necessarily encoded in their biosynthetic gene clusters. Traditionally the mature peptide resulting from the transcription of *lanA1* is referred as the α- peptide and that from *lanA2* as the β-peptide.

1.2.3 Biosynthetic elements

1.2.3.1 Prepropeptides

As previously described, the structural *lanA* genes encode the precursor of the mature lantibiotic – the prepropeptide (Willey and van der Donk, 2007). The isolation of these prepropeptides from the cytoplasm of the producing strain has been difficult, suggesting that they have a short half-life, possibly being dehydrated immediately after their synthesis.

Prepropeptides are biologically inactive and can be dissected into the N-terminal leader sequence and the C-terminal propeptide regions (McAuliffe *et al.*, 2001b). Both of these regions contain Ser and Thr residues, while cysteines have been found exclusively in the propeptide segment (Chatterjee *et al.*, 2005). Though, only the Ser and Thr residues present in the propeptide are dehydrated to Dha and Dhb, respectively. The production of a fully modified and active (mature) lantibiotic also implies the proteolytic removal of the leader sequence. Thus, the first amino acid of the active peptide will be coincident with the first amino acid of the propeptide. That is why the enumeration of the amino acids constituting the leader sequence is performed with negative numbers, having the proteolytic position as a starting point.

Leader Sequences

The lantibiotics' leader sequences do not have homology with the *sec*-dependent transport signal sequences since they lack a hydrophobic membrane-spanning core and their typical processing site (McAuliffe *et al.*, 2001b). An exception was identified in the

leader peptide of the class II lantibiotic cinnamycin, which possesses the AXA motif required for the activity of type I signal peptidases of the general secretory *sec*-pathway (Widdick *et al.*, 2003).

The leader sequences of class I lantibiotics are about 25 amino acids long and are normally rich in Asp residues (Oman and van der Donk, 2010). The alignment of these sequences allowed the identification of a common FNLD motif between the positions -20 and -15 and usually a Pro in the position -2 (Chatterjee *et al.*, 2005) (Figure 5). The importance of this consensus sequence was shown after introduction of mutations that resulted in the completely abolishment or reduction of the mature lantibiotics nisin and pep5 production (van der Meer *et al.*, 1994; Neis *et al.*, 1997).

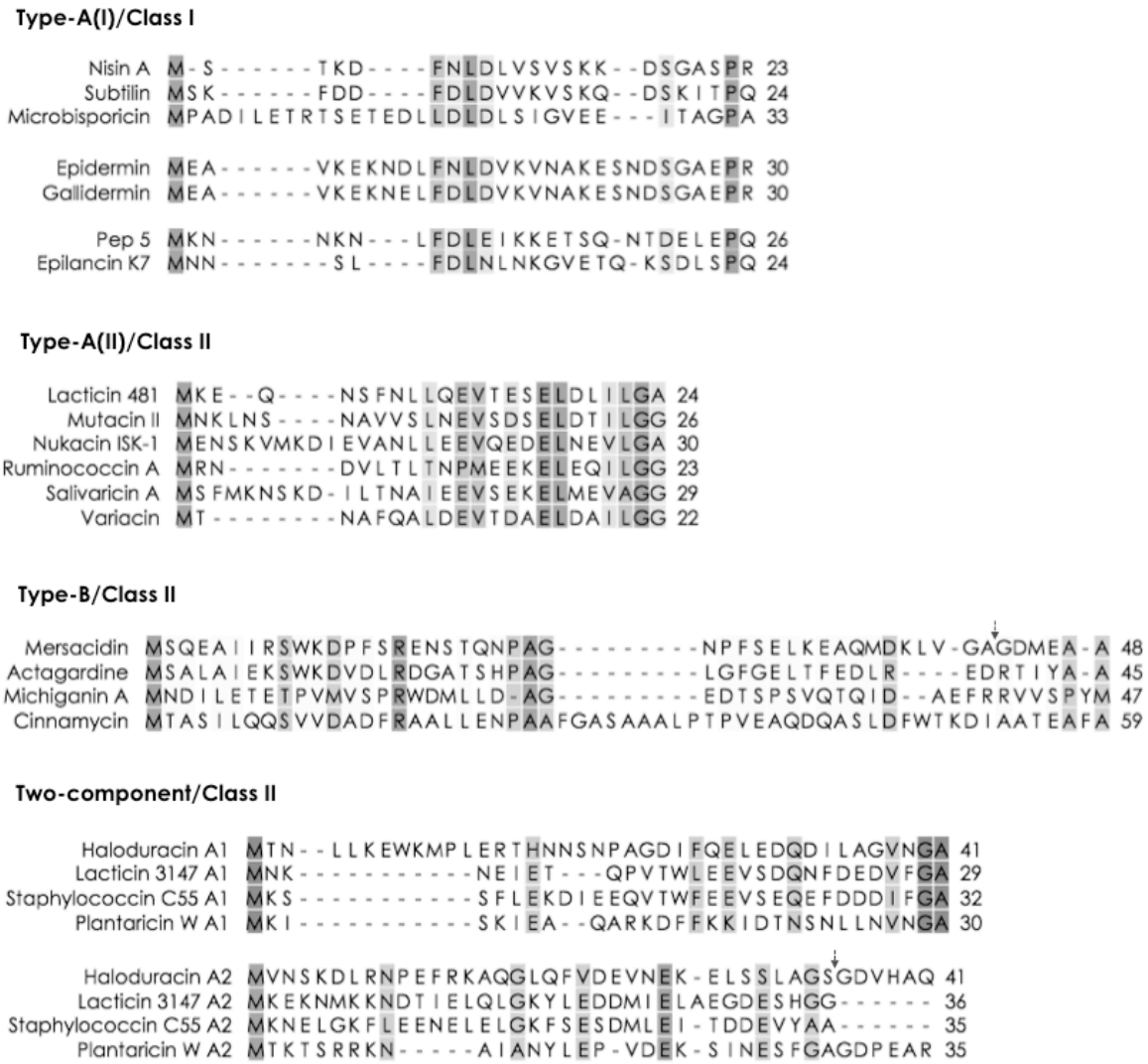


Figure 5: Alignment of the leader peptide sequences from the prepropeptides of the most characteristic lantibiotics from each group. Conserved amino acids are highlighted in grey shades. The dashed arrow represents the cleavage of mersacidin, haloduracin A2 and plantaricin W A2 leader sequences.

The leader sequences of the class II lantibiotics are generally longer than those of class I, and they are typically rich in Asp (similarly to those of class I) and Gln residues (Oman and van der Donk, 2010). The C-terminus of these leader sequences is characterized by the presence of the conserved amino acid sequence GlyGly, GlyAla or GlySer/Thr, historically refereed as the "double Gly motif" (Figure 5). This motif is essential for the leader peptide proteolytic removal process (section 1.2.3.4, page 22).

The importance of the leader sequence for the biosynthesis of lantibiotics has been largely discussed. It was demonstrated both *in vivo* and *in vitro* that an efficient dehydration and cyclization of the nisin and lacticin 481 propeptides requires their leader peptides (Kluskens *et al.*, 2005; Li *et al.*, 2006; Patton *et al.*, 2008). Nevertheless, it has been difficult to delineate the exact factors involved in its recognition by the modifying enzymes, since almost all the single point mutants of the lacticin 481 leader peptide were still processed *in vitro* by the LctM enzyme (Xie *et al.*, 2004; Oman and van der Donk, 2010). It has also been observed that the fully modified propeptides attached to their leader sequences present low or none antimicrobial activity. Thus, it is proposed that the leader sequences are part of a protective mechanism of the producer strain, keeping the fully modified propeptide inactive until it is safely outside the cell (van der Meer *et al.*, 1994; Xie *et al.*, 2004; Li *et al.*, 2006). Another hypothesis considers that the leader peptides provide a signal for the transport. This is supported by the fact that some non-lantibiotic peptides attached to the nisin and lacticin 3147 LtnA2 leader sequences were successfully translocated to the producers' supernatant (Kuipers *et al.*, 2004; Kuipers *et al.*, 2008).

Propeptides and structures of mature lantibiotics

As abovementioned, the mature lantibiotic sequence and structure results from the post-translational modification of the propeptide residues. Type-A propeptides normally possess 34 residues, whereas type-B are generally smaller (up to 19 residues) (McAuliffe *et al.*, 2001b). Despite the fact that propeptides are synthesized by the ribosome, the composition of their mature forms is not restricted to the 20 proteinogenic amino acids as already confirmed by the presence of the characteristic Dha, Dhb, Lan and MeLan amino acids. In fact, at least more than 11 different modifications were already described (Figure 6). For instance, the Cys residues present in the C-terminus of the propeptide can form *S*-aminovinyl-D-cysteine (AviCys) or *S*-aminovinyl-3-methyl-D-cysteine (AviMeCys) structures. Also, Dha and Dhb residues that are N-terminally exposed, after the proteolysis of the leader sequence, can spontaneously hydrolyze to yield the 2-oxopropionyl (OPr) and the 2-oxobutyryl (OBu) residues as observed with pep5 lantibiotic (Willey and van der Donk,

2007). Moreover, similarly to the non-ribosomal peptides, lantibiotics can possess D-amino acids, since L-Ser can be converted to D-Ala, having a Dha residue as an intermediate. This is a characteristic of the lacticin 3147 lantibiotic (Cotter *et al.*, 2006b).

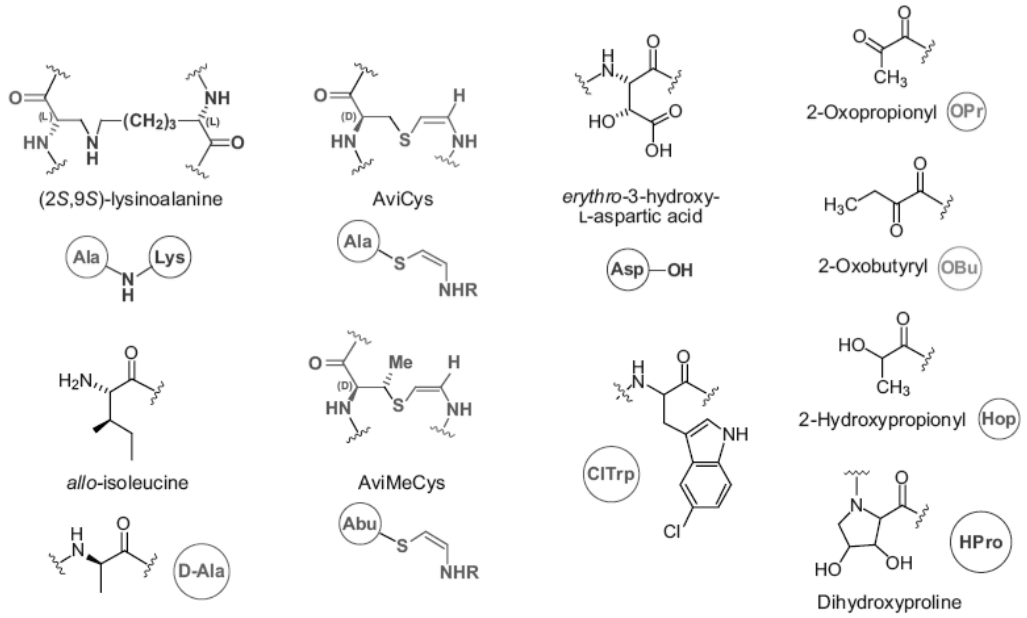


Figure 6: Some posttranslational modifications described in lantibiotics. Adapted from Willey and van der Donk (2007).

The structures and unique characteristics acquired by propeptides after the intervention of the modifying enzymes allow their clustering in at least seven groups (Figure 7 and Table 1): i) the nisin, epidermin and pep5 groups of type-A(I), ii) the lacticin 481 group of type-A(II), iii) the mersacidin and cinnamycin groups of type-B and iv) the lacticin 3147 group, comprising the two-component lantibiotics (Chatterjee *et al.*, 2005). The last group is characterized by the synthesis of two peptides, which are traditionally designated by α- and β-nomenclature after their full maturation process. Characteristically, the α-peptides belong to the type-B lantibiotics, whereas the β-peptides resemble the type-A lantibiotics (Bierbaum and Sahl, 2009). The bioengineering and structure activity relationship (SAR) studies of some of these lantibiotics have been performed by the introduction of mutations in the propeptide sequence. Several *in vivo* and *in vitro* systems for this propose were already established for lantibiotics belonging to all of these seven groups (Cortes *et al.*, 2009; Field *et al.*, 2010).

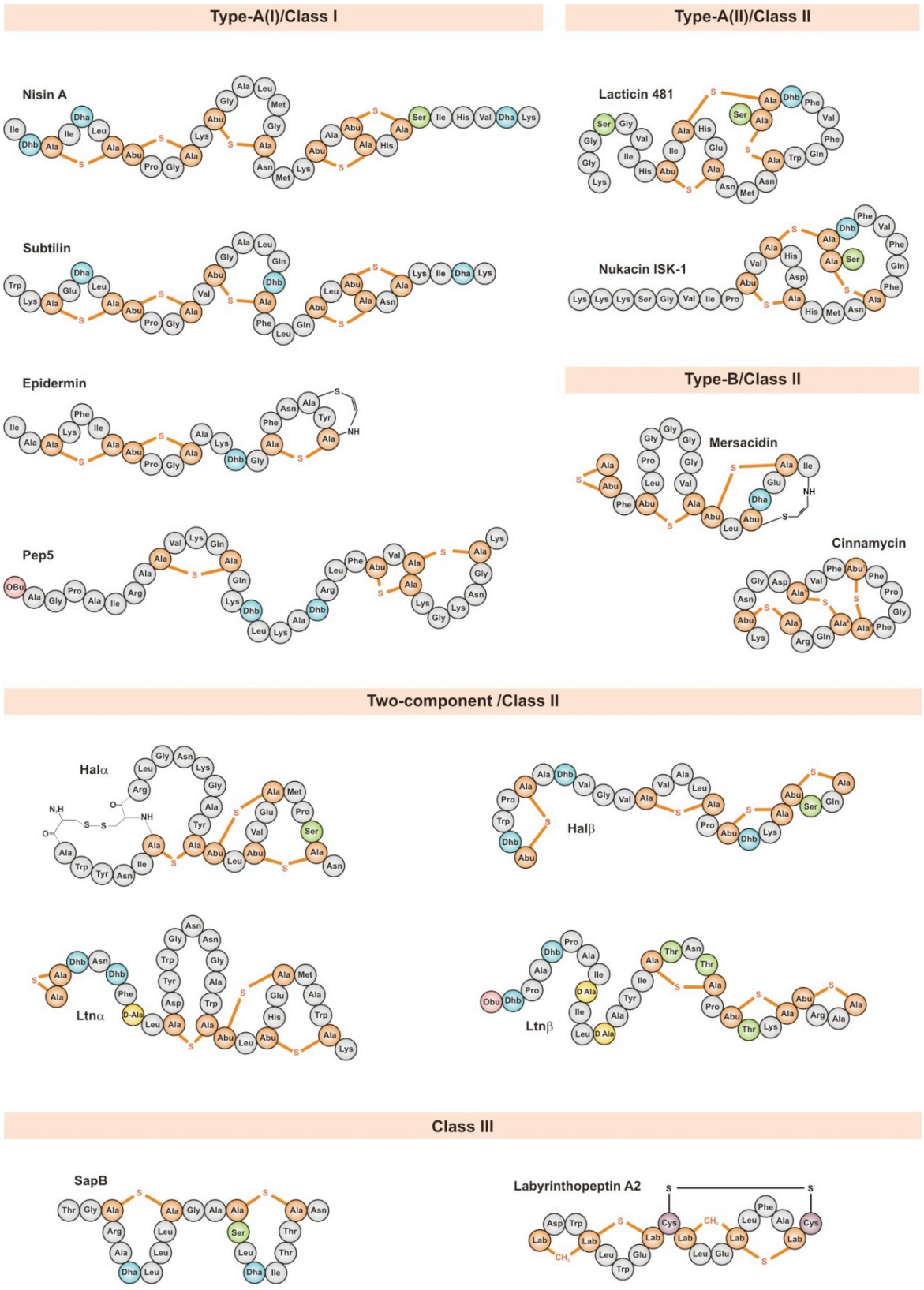


Figure 7: Representative examples of lantibiotics grouped according to their structural and/or biosynthetic pathway.

1.2.3.2 Dehydratases and cyclases enzymes

The dehydration of Ser and Thr residues in the LanA propeptide is one of the key reactions in the biosynthesis of lantibiotics, followed by the formation of the Lan and MeLan thioether rings. In Class I lantibiotics such modification is carried out by the LanB dehydratases and the LanC cyclases, respectively. For class II lantibiotics, the bifunctional enzyme LanM exhibits these two catalytic functions. In the recent years, a remarkable progress concerning the biochemistry of the modification reactions has been achieved, especially due to the *in vitro* reconstitution of lacticin 481 and haloduracin LanM and nisin LanC activities (Bierbaum and Sahl, 2009).

Dehydratases of Class I lantibiotics

LanB dehydratases are proteins containing approximately 1000 amino acid residues and possessing about 120 KDa of molecular weight, which do not reveal homology with any other known proteins (McAuliffe *et al.*, 2001b; Chatterjee *et al.*, 2005). Among the LanB family, the overall sequence identity is merely around 30 %. Nevertheless the homology increases between enzymes of structurally related lantibiotics (Chatterjee *et al.*, 2005). The rather hydrophobic nature of these enzymes, associated with the presence of some hydrophobic domains suggested their association with the cytoplasmic membrane (McAuliffe *et al.*, 2001b). This has been confirmed for the nisin and subtilin LanB proteins, while a loose association with the cytoplasmatic membrane was observed for the epidermin LanB protein (Engelke *et al.*, 1992; Gutowski-Eckel *et al.*, 1994; Peschel *et al.*, 1996).

The first evidences that LanB proteins are involved in Ser and Thr dehydration were provided for the biosynthesis of pep5 and epidermin (Meyer *et al.*, 1995). Evidence of such functionality was obtained by studies involving the expression and purification of His-tagged nisin precursors in knockout mutant strains lacking the nisin dehydratase and cyclase, NisB and NisC, respectively. This strategy demonstrated that nisin precursors were totally unmodified in the absence of NisB and dehydrated but devoid of normal lanthionine formation when NisC was not present (Koponen *et al.*, 2002). Regarding the substrate specificity of LanB proteins, it has been shown that it is rather flexible. For instance it was demonstrated that NisB is able to dehydrate Ser and Thr residues of non-lantibiotic compounds such as the human peptide hormones if they are N-terminally fused to the nisin leader peptide (Kluskens *et al.*, 2005). The same study also revealed that NisB is active in the absence of NisC. Likewise, Kuipers and co-workers (2004) demonstrated that NisT presence was also not essential for the NisB activity. Additionally, it was also demonstrated

that the distance between the Ser and Thr residues and the nisin leader sequence should not influence the NisB dehydration activity (Kluskens *et al.*, 2005).

Information concerning the mechanistic of lantibiotic-enzyme mediated dehydration is not currently available for LanB proteins. Related to this aspect, the *in vitro* reconstitution of NisB, or other LanB synthetases, will be a major achievement.

Cyclases of class I lantibiotics - LanC

The formation of Lan and MeLan in Class I lantibiotics has been addressed to the LanC cyclases. These proteins are constituted by approximately 400 residues and show no sequence similarity with known proteins (Nagao *et al.*, 2006). The sequence homology among LanC proteins is low (20-30 %). Nonetheless, they share a number of structural motifs, including GXAHG, WCXG and CHG, in which the His and Cys amino acids are conserved (McAuliffe *et al.*, 2001b). The hydrophobicity plots of LanC proteins revealed the presence of alternate hydrophobic and hydrophilic regions, suggesting also a membrane location for these proteins (Engelke *et al.*, 1992; McAuliffe *et al.*, 2001b). In fact this was demonstrated for NisC, whereas the epidermin cyclase EpiC could not be detected in membrane fractions of its producer strain (Kupke and Götz, 1996; Lubelski *et al.*, 2008).

The first experimental evidences of LanC functionality was obtained with the pep5 biosynthetic system, where the disruption of its cyclase gene (*pepC*) led to the formation of a fully dehydrated prepropeptide (as well as some prepropeptide fragments) but without the correct formation of Lan and MeLan bridges (Meyer *et al.*, 1995). Yet, this study also established that thioether bridges could occur spontaneously. This is explained by the fact that the cyclization of a nucleophilic thiolate onto an electrophilic 2,3-didehydro amino acid is expected to be a relatively fast and easy reaction (Chatterjee *et al.*, 2005). However, the occasional occurrence of such spontaneous reactions does not substitute the regio- and stereochemistry of LanC activity. Regarding the directionality of processing, LanC cyclases incorporate the lanthionine rings solely in the N-to-C terminal, i.e., the cysteine involved in the cyclization is always located C-terminally of its Dha or Dhb reaction partner (Kaletta *et al.*, 1991; Chatterjee *et al.*, 2005). The *in vitro* reconstitution of NisC was presented by van der Donk and co-workers (2006). In this system, the NisC enzyme successfully mediated the cyclization of the previously dehydrated nisin prepropeptide. Moreover, after the removal of the leader sequence the *in vitro* produced peptide possessed antimicrobial activity. This study also confirmed that nisin leader

sequence is essential for the binding and recognition of the prepropeptide by NisC (Li *et al.*, 2006).

NisC was the first lantibiotic cyclase to be crystallized (Li *et al.*, 2006). The analysis of its structure together with the previous results and mutagenesis studies on NisC and SpaC (subtilin LanC) enzymes allowed the recognition of some residues which are involved in the activation of the thiolate for nucleophilic attack as well as some residues that should function as catalysts of the ring formation (Okeley *et al.*, 2003; Li *et al.*, 2006; Helfrich *et al.*, 2007; Li and van der Donk, 2007). The structure of the NisC enzyme also indicated the presence of a possible binding site for the nisin leader sequence. This putative binding groove is located next to the catalytic site and is lined with a number of hydrophobic and negatively charged residues forming a channel in which the positively charged leader sequence is presumably trapped (Li *et al.*, 2006; Lubelski *et al.*, 2008).

Bifunctional dehydro-cyclases of class II lantibiotics- LanM

In Class II lantibiotics the dehydration and cyclization reactions are performed by the bifunctional enzymes designated LanM. These proteins possess 900 to 1000 amino acids and molecular weights of approximately 120 KDa. Their C-terminus contains 20-27% sequence identity with LanC proteins. However, no identity was found with LanB enzymes (Chatterjee *et al.*, 2005; Nagao *et al.*, 2006). Thus it was assumed that the N-terminal domain of LctM should be responsible for the dehydration reaction of Ser and Thr residues, while the C-terminal should catalyze the Lan and MeLan ring formation (You and van der Donk, 2007).

The first evidences of LanM catalytic activity were obtained through the disruption of *lanM* genes in several biosynthetic clusters, including those of lacticin 481, mutacin II and lacticin S, which always resulted in the abolishment of the mature lantibiotic production (Gilmore *et al.*, 1994; Skaugen *et al.*, 1997; Woodruff *et al.*, 1998; Uguen *et al.*, 2000). Direct proof of such functionality was obtained with the first *in vitro* reconstitution of the LanM enzyme activity. The study was performed with the lacticin 481 LctM dehydro-cyclase, in which a 4-fold dehydrated peptide harboring the correct cyclization pattern and antimicrobial activity was obtained (Xie *et al.*, 2004). Later, it was shown that the cyclization reaction occurs on a similar time scale as dehydration, since some not fully dehydrated intermediates also possess thioether rings within their N-terminal propeptide regions (Lee *et al.*, 2009). The leader sequence is generally important for substrate recognition. Unexpectedly, the incubation of LctM just with the lacticin 481 propeptide resulted in some dehydration, suggesting that this enzyme has some basal activity in the

absence of the leader sequence. It was also shown that dehydration also occurs when the LctA propeptide is C-terminally fused with its leader peptide, albeit with much decreased efficiency. Also, in that situation the dehydration process was non-directional (Levengood *et al.*, 2007). Recently, the dehydration of Ser and Thr residues was shown to be directionally performed from the N- to the C-terminus of the lacticin 481 and the haloduracin HalA2 prepropeptides (Lee *et al.*, 2009). Therefore, the presence of the leader sequence in the propeptide N-terminal is important for the occurrence of such directionality. Similarly, the reactions leading to the formation of the thioether rings was also demonstrated to occur from the N- to the C-terminus of the HalA2 propeptide (Lee *et al.*, 2009). The attempts to characterize LanM proteins through crystallography have not yet been successful. However, site directed mutagenesis studies of the LctM enzyme have shed light on important residues for the dehydration and cyclization activities of LanM synthetase families and a model was proposed that assigns a role to each of these residues (Paul *et al.*, 2007; You and van der Donk, 2007).

In vivo studies have shown that LanM has a high tolerance for amino acid changes in the propeptide region, since several processed lantibiotic variants were already obtained (Chatterjee *et al.*, 2005; Alain *et al.*, 2007). The relatively relaxed substrate specificity of LanM enzymes was shown to be more extensive since LctM demonstrated its ability to modify the propeptides of nukacin ISK-1, mutacin II and ruminococcin A lantibiotics, when they were N-terminally fused to the lacticin 481 leader sequence in *in vitro* assays (Patton *et al.*, 2008). In the same way, synthetic non-lantibiotic peptides containing both proteinogenic and non-proteinogenic amino acids were also successfully modified by LctM in the same conditions (Levengood and van der Donk, 2008; Levengood *et al.*, 2009).

1.2.3.3 Other post-translational modifications

As already mentioned, some lantibiotics possess other post-translational modifications besides the presence of dehydro amino acids and the thioether rings. The lantibiotics epidermin (class I) and mersacidin (class II) possess the unusual amino acids AviCys and AviMeCys, respectively, at their C-terminus (Figure 7, page 15). These amino acids are the product of the oxidative descarboxylation of the Cys residue at the C-terminus. It was shown that this reaction is catalyzed *in vitro* by the EpiD and MrsD enzymes for the epidermin and mersacidin lantibiotics, respectively (Kupke *et al.*, 1992; Majer *et al.*, 2002). The conversion of the L-Ser residues to D-Ala residues in the two lacticin 3147 peptides was shown to be catalyzed by LtnJ enzyme. It has been suggested that LtnJ protein should act

after the dehydration of the Ser residues by LtnM (Cotter *et al.*, 2005b). All the genes encoding these proteins were successfully detected in the respective lantibiotic biosynthetic cluster. More recently, the presence of a sulfoxide group in the unique lantibiotic actagardine (class II) was shown to be catalyzed by the luciferase-like monooxygenase GarO, encoded in its biosynthetic cluster (Boakes *et al.*, 2009).

Several other modifications can be found in mature lantibiotics, however the enzymes involved in such process are still unknown.

1.2.3.4 Proteases and transporters

The translocation of lantibiotics across the cytoplasmatic membrane does not involve the use of the general secretory pathway (McAuliffe *et al.*, 2001b) and their biological activity is dependent on the removal of the leader sequence. For Class I lantibiotics these functions are exerted by two distinct enzymes: LanP and LanT, respectively. The Class II lantibiotics, a single multifunctional enzyme carries out the catalysis of both reactions.

Leader peptide processing of class I lantibiotics - LanP

LanP are proteins with a wide variety of sizes and all of them share homology with the serine protease subtilisin. Their size is dependent upon the presence or absence of two sequences: a N-terminal *sec*-signal and a C-terminal cell wall anchor motif (Chatterjee *et al.*, 2005). The presence of these motifs either suggests the excretion of LanP proteins to the extracellular environment or their functionality as membrane anchors. Both of these motifs are present in the nisin protease NisP. Therefore, the leader sequence of the modified nisin prepropeptide should be removed after its transport and in the producer cell surface. Indeed, it was shown that intact cells of the nisin producer *L. lactis* NZ9800 and intact cells of *E. coli* expressing the NisP enzyme were able to remove the leader sequence of a modified nisin prepropeptide. On the contrary, their cell-free supernatants or their membrane-free extracts did not exhibit such proteolytic activity (van der Meer *et al.*, 1993). However, this does not constitute the rule among class I lantibiotics. For instance, the proteolytic activity of the epidermin EpiP (which lacks the cell wall anchor motif) was detected in the supernatant of the producer strain (Geissler *et al.*, 1996). Proteases belonging to other lantibiotics' biosynthetic pathways do not harbor any of the abovementioned N- or C-terminus, suggesting their intracellular location. Examples include the ElkP, PepP and LasP encoded in the epilancin K7, pep5 and lactocin S gene clusters, respectively.

Despite the importance of the leader sequence removal for the lantibiotics bioactivity, the identification of a *lanP* gene among the lantibiotics' gene clusters it is not always possible, suggesting that a variety of secreted proteases of the producer strain can also play this role. This is the case of subtillin, produced by *B. subtilis*, whose leader sequence is processed by proteins present in the producer supernatant, most probably by subtilisin (AprE), WprA or Vpr proteases (McAuliffe *et al.*, 2001b; Corvey *et al.*, 2003).

The studies involving the substrate requirements of LanP enzymes suggest that the Arg-1 residues (Figure 5 on page 12) of NisP and EpiP are essential for their proteolytic activities (Kuipers *et al.*, 1993b; van der Meer *et al.*, 1994; Siezen *et al.*, 1995). Moreover, it was found that the formation of Lan and MeLan rings are essential for their activity, since dehydrated but linear NisA and PepA peptides were not substrates *in vivo* for the NisP and PepP proteases (Meyer *et al.*, 1995; Kuipers *et al.*, 2004).

Transport of class I lantibiotics - LanT

The LanT proteins are about 600 amino acids in length and bear significant homology to hemolysin B-like ATP-dependent transport proteins present in a wide variety of organisms. The LanT proteins are generally characterized by one hydrophobic N-terminus, a six-helix membrane spanning domain and a C-terminus ATP binding domain (Chatterjee *et al.*, 2005). However, a typical ABC-transporter should include two of each of these domains. Therefore, LanT is normally considered a half-transporter, which will most likely require another half-transporter to form an active unit. For the nisin NisT, the putative partner is suggested to be another molecule of NisT (Lubelski *et al.*, 2008).

For some lantibiotic systems, the LanT transporter encoded in the biosynthetic gene cluster is not essential for the lantibiotic translocation to the extracellular environment. This was observed for the pep5 lantibiotic where the deletion of its transporter PepT still resulted in the presence of the lantibiotic in the producer supernatant, despite a 10 % reduction in its production rate. Therefore, the PepT functionality could be partially replaced by other host-encoded transporters (Meyer *et al.*, 1995). Controversially, the deletion of the *nisT* gene in the nisin producer strain led to the accumulation of nisin in the cytoplasm, while extracellular nisin could not be detected (Qiao and Saris, 1996).

Regarding the substrate specificity of LanT transporters, it should be quite relaxed, since NisT was able to export unmodified nisin prepropeptides as well as non-lantibiotic peptides N-terminally fused to the nisin leader sequence (van den Berg van Saparoea *et al.*, 2008). This also proved that the transport of nisin by NisT is not dependent on the presence of the Lan and MeLan thioether rings. Additionally, the NisT was able to secrete the fully modified

nisin Z propeptide N-terminally fused to the leader sequence of the subtilin lantibiotic produced by *B. subtilis* (Kuipers *et al.*, 1993b). Consequently, either the leader sequence is not essential for the lantibiotic translocation or the residues involved in this process are conserved among nisin and subtilin leader sequences.

Leader peptide processing and transport of class II lantibiotics - LanT

The LanT proteins encoded in the biosynthetic clusters of Class II lantibiotics are about 700 residues long. These are characterized by an N-terminal peptidase domain (not present in class I LanT proteins), an intermediary membrane spanning motif and the C-terminus ATP-binding domain (Chatterjee *et al.*, 2005). LanT proteins and transporters of class II bacteriocins (without posttranslational modifications) exhibit a degree of homology between them. Such transporters are able to process the N-terminal leader sequence and export the bacteriocins (Havarstein *et al.*, 1995). The leader sequences of these bacteriocins also present significant similarity with those of class II lantibiotics such as lacticin 481, mutacin II and variacin, including the double Gly type cleavage site (Chatterjee *et al.*, 2005). For these reasons, it was deduced that LanT proteins would display the same dual functions: i) proteolytic removal of the prepropeptide leader sequence and ii) translocation of the lantibiotic across the cytoplasmatic membrane (Uguen *et al.*, 2005). By analogy, the double Gly motif (GG/GA/GS) classically present at the C-terminus of the leader sequences of class II lantibiotics was considered the substrate for the LanT N-terminal catalytic activity.

The first studies involving LanT enzymes were performed *in vivo* for the mutacin II and lacticin 481 transporter/proteases MutT and LctT, respectively (Rince *et al.*, 1994; Chen *et al.*, 1999). It was described that in the absence of MutT, no antibacterial activity could be detected in the mutacin II producer supernatant (Chen *et al.*, 1999). Identical results were obtained for nukacin ISK-1 producer upon the deletion of the NukT-encoding gene (Aso *et al.*, 2004a). On the contrary, the inactivation of the *lctT* gene in the lacticin 481 producer was not accompanied by the abolishment of antibacterial activity. Such results suggested that LctT enzyme was dispensable for the lacticin 481 production (Rince *et al.*, 1994). However, it was further demonstrated that the antagonistic activity was due to a smaller lacticin 481 derivative. Consequently, it was proved that the LctT enzyme is responsible for the specific cleavage of lacticin 481 leader sequence at the GG motif and that its functionality as transporter can be substituted by other host proteins (Uguen *et al.*, 2005).

The activity of the LctT N-terminal protease domain (150 amino acids) was successfully reconstituted *in vitro* by Ihnken *et al.* (2008). The proteolysis of lacticin 481 leader sequence

in vitro undoubtedly showed that the LanM transmembrane domain is not required for the substrate recognition and proteolysis. Recently, the peptidase activity of the full-length NukT was also demonstrated by an *in vitro* assay (Nishie *et al.*, 2009). The outcome of both studies revealed that the LctT N-terminus protease domain and the NukT enzyme have distinct requirements with respect to the formation of Lan and MeLan rings prior to proteolysis. LctT N-terminal protease was able to remove the lacticin 381 leader sequence without the presence of its thioether rings, whereas NukT was not able to process the nukacin ISK-1 prepropeptide in the same conditions (Ihnken *et al.*, 2008; Nishie *et al.*, 2009).

Some studies involving the mutagenesis of the double GG motif confirmed that it is essential for the *in vivo* proteolysis of class II leader sequences (Chen *et al.*, 2001). This was also confirmed for the *in vitro* proteolysis activity of the LctT N-terminal (Ihnken *et al.*, 2008). Also, it was shown that the modifications of the lacticin 481 propeptide after the GG cleave site residues did not influence the LctT peptidase activity *in vitro*. Though, the disturbance of the leader sequence helical structure seemed to affect its functionality (Ihnken *et al.*, 2008).

The flexible activity of LanT enzymes *in vivo* has also been described. For instance, several variants of class II lantibiotics such as mersacidin and lacticin 3147 were successfully processed and transported by their producer strains (Cooper *et al.*, 2008; Appleyard *et al.*, 2009). More surprisingly, it was possible to obtain the fully modified lacticin 481 lantibiotic in the host's extracellular environment, using the co-expression of the lacticin 481 prepropeptide with the nukacin ISK-1 biosynthetic machinery, without any leader sequence fusion (Nagao *et al.*, 2007).

1.2.4 Regulation of biosynthesis

Synthesis and export of lantibiotics are energetically demanding processes and consequently have to be closely controlled (Bierbaum and Sahl, 2009). Indeed, proteins that are involved in such processes are encoded in some gene clusters of class I and class II lantibiotics. The regulatory systems identified so far consist of two protein components: the receptor-histidine kinase LanK, which monitors an environmental signal, and the transcriptional response regulator LanR (McAuliffe *et al.*, 2001b). It has been proposed that in response to an external environmental signal, the LanK protein (most probably present on the cell surface) initiates a signal cascade by the autophosphorylation of a histidine residue. During this cascade, the phosphoryl group should be transferred to a conserved Asp residue of LanR, that is intracellularly located, and which will then mediate the final adaptative response (usually a change in gene

expression) (McAuliffe *et al.*, 2001b; Chatterjee *et al.*, 2005). A general overview of this process is represented in Figure 8.

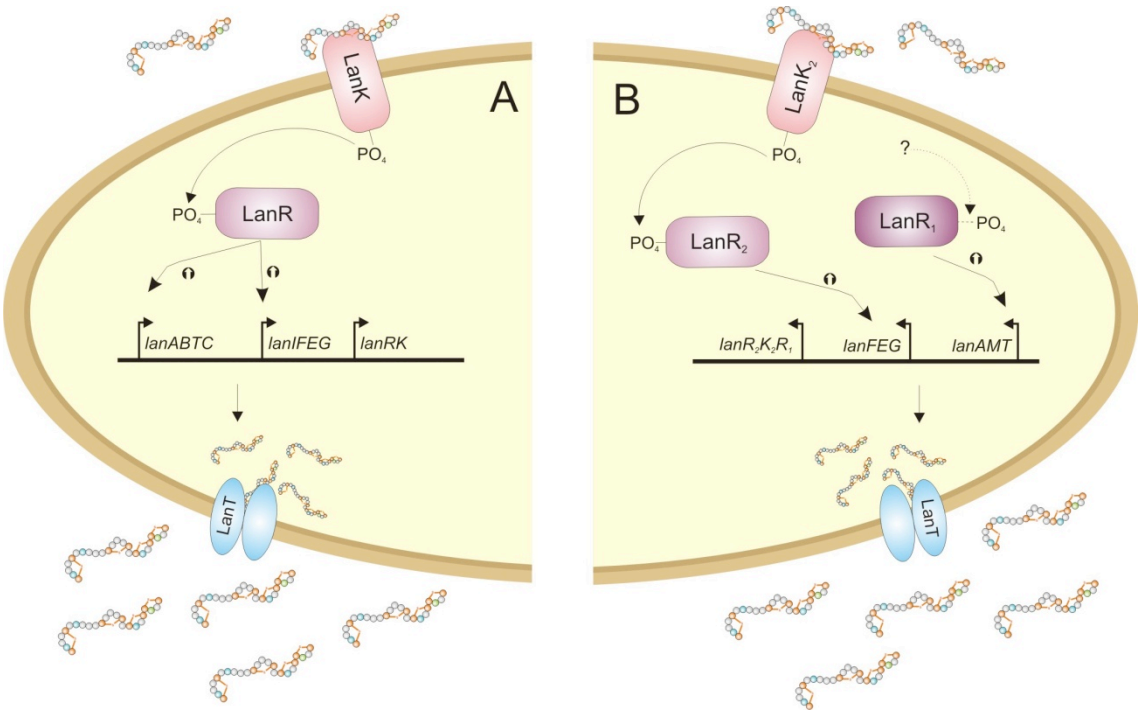


Figure 8: General overview of the main systems involved in the regulation of lantibiotics. A represents the systems in which the transcription of the structural gene and immunity genes are up-regulated by the same two-system LanKR, triggered by the mature lantibiotic itself. This type of regulation occurs during the biosynthesis of lantibiotics such as nisin and subtilin. B represents those systems in which the transcription of the structural gene and immunity genes are not up-regulated by the same proteins. In lantibiotics such as mersacidin, the regulation of the structural gene is normally controlled by a LanR protein with a non-assigned histidine-kinase whereas the transcription of the immunity genes is controlled by a two-system LanKR, also triggered by the mature lantibiotic.

It has been shown that the production of the lantibiotics nisin, subtilin, salivaricin, bovicin HJ50, ruminococcin A and mersacidin are controlled by such two-component regulation systems (Klein *et al.*, 1993; Engelke *et al.*, 1994; Altena *et al.*, 2000; Gomez *et al.*, 2002; Wescombe *et al.*, 2006; Ni *et al.*, 2011). For nisin, subtilin, bovicin HJ50 and salivaricin, but not for ruminococcin A, the lantibiotic itself acts as the sensory molecule that triggers the transcription of their prepropeptides and self-protection determinants (Bierbaum and Sahl, 2009). For instance, in the case of nisin, as few as five extracellular molecules are sufficient to activate the transcription of *nisABTCIP* operon involved in its biosynthesis as well as of *nisFEG* involved in the self-immunity of the producer strain (Kuipers *et al.*, 1995; de Ruyter *et al.*, 1996). The investigation of nisin regulation mechanism also demonstrated that other class I lantibiotics such as subtilin and pep5 are not able to induce the NisK kinase, neither

the unmodified nisin, highlighting the stringent substrate specificity of NisK (Kuipers *et al.*, 1995). Even so, the subtilin histidine-kinase SpaK was able to phosphorylate NisR in the presence of subtilin (Kleerebezem, 2004). Moreover, Kuipers *et al.* (1995) proved that the transcription of a determined reporter gene fused to the *nisA* promoter could be induced in the presence of nisin and its regulatory components NisK and NisR. Such results led to the development of a heterologous-controlled protein expression system in *L. lactis* designated as NICE (nisin-controlled expression) (de Ruyter *et al.*, 1996; Kuipers *et al.*, 1997; Eichenbaum *et al.*, 1998). This system has been successfully used for the overexpression of a variety of proteins including the notoriously difficult-to-overproduce membrane proteins (Lubelski *et al.*, 2008).

The regulation of production of the class II lantibiotic mersacidin was shown to involve the two-component system MrsK2/MrsR2 and the single regulatory protein MrsR1. The mersacidin producer *Bacillus* sp. HIL Y-85,54728 uses the MrsK2/MrsR2 tandem to activate the transcription of the immunity genes, whereas the MrsR1 protein is responsible for promoting the biosynthesis of the lantibiotic (Guder *et al.*, 2002). The addition of mersacidin to exponentially growing cultures (before the onset of mersacidin biosynthesis) markedly increased the transcription of its structural gene *mrsA*. Therefore, alike nisin, mersacidin is also able to regulate its own synthesis, whereas relatively high concentration of this lantibiotic were required for the process (Schmitz *et al.*, 2006). It was not clear whether MrsR1 needs phosphorylation to exert its activity, since a dedicated kinase was not identified (Guder *et al.*, 2002). Nevertheless, the same situation have been described for other lantibiotics including epidermin (Peschel *et al.*, 1993) and mutacin II (Qi *et al.*, 1999a).

The systems described before involve the activation of transcription. Nevertheless, repressors of gene expression were also characterized for the two-component lantibiotics lacticin 3147 and cytolysin (McAuliffe *et al.*, 2001a; Haas *et al.*, 2002). In the lacticin 3147 producer it was found that LtnR was able to bind to the promoter responsible for the transcription of the self-protection operon (*ltnRIFE*), significantly reducing its expression (McAuliffe *et al.*, 2001a). Repression of the exotoxin cytolysin biosynthetic machinery by the CylR1 and CylR2 proteins was also described (Haas *et al.*, 2002). Interestingly, in this biosynthetic system it was showed that the both cytolysin peptides were produced at basal levels and could associate to form an inactive complex. However, in the presence of their target cells, the complex dissociates and one of the peptides ($CylL_S$) preferentially binds to the lipid bilayer of the eukaryotic cells, promoting the accumulation of the other peptide ($CylL_L$). Subsequently $CylL_L$ peptide can trigger the cytolysin regulation

mechanism leading to the depression of synthesis via CylR2 binding to the cytolysin promoter region (Haas *et al.*, 2002; Rumpel *et al.*, 2004).

Other systems have been identified that regulate the lantibiotics' synthesis, which are not encoded in their biosynthetic clusters. Examples include the induction of lacticin 418 production by pH decrease, via the RcfB protein (Madsen *et al.*, 2005), whereas ruminococcin A production can be triggered by high cell density (Gomez *et al.*, 2002).

1.2.5 Lantibiotics' antibacterial mode of action

In general, class I and class II lantibiotics' are bactericidal against a variety of Gram-positive bacteria at nanomolar concentrations and some are active against methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci (VRE) and oxacillin-resistant Gram-positives (Willey and van der Donk, 2007).

The type-A(I) lantibiotics such as nisin are characterized by a dual mode of action, targeting the cell-wall biosynthesis as well as the integrity of the cytoplasmic membrane (Wiedemann *et al.*, 2001). Pore formation is a widespread property of bactericidal antimicrobial peptides produced by eukaryotes, such as the magainins (isolated from frog's skin). Therefore, much of the initial work on the nisin mode of action involved the investigation of nisin aptitude in the permeabilization of bacterial membranes through the generation of pores (Chatterjee *et al.*, 2005). In fact, several studies confirmed that nisin has such ability. However, it was noticed that the nisin MIC against intact cells was within the nM range, whereas much higher concentrations were required to induce pore formation in model membranes, suggesting that other nisin-bacteria interactions should contribute for the antibacterial activity of nisin. Indeed, it was shown that nisin could also interact in a highly specific manner with the lipid II molecule (Brötz *et al.*, 1998; Breukink *et al.*, 1999). Lipid II is a membrane-anchored molecule that is the main vehicle for transport of peptidoglycan subunits from the cytoplasm to the bacterial cell-wall. Thus its binding to nisin prevents the correct cell wall synthesis, contributing to cell death (Cotter *et al.*, 2005a; Breukink and de Kruijff, 2006). Furthermore, it has been proposed that nisin exerts its biological activity using lipid II as a docking molecule for pore formation (Bierbaum and Sahl, 2009). In this process, the two N-terminally located thioether rings should form a binding pocket (referred as the pyrophosphate cage) that envelops the undecaprenyl pyrophosphate moieties of the lipid intermediates of cell wall biosynthesis (Hasper *et al.*, 2004; Hsu *et al.*, 2004). After binding, the highly positively charged C-terminus of nisin should interact with the anionic membrane surface and should be able to insert into the membrane, oligomerize and form a pore (Figure 9) (Breukink *et al.*, 2003; Hsu *et al.*, 2004; Bierbaum and Sahl, 2009). However, in other type-A(I) lantibiotics such as epidermin,

mutacin 1140 and gallidermin, which are shorter than nisin, the ability to induce a pore in the cytoplasmic membrane is believed to depend on the thickness of the bacterial wall, which should not exceed more than 40 Å (Bonelli *et al.*, 2006; Smith *et al.*, 2008). Therefore, for the type-A(II) lantibiotics it is believed that they can act by complexing with lipid II optionally followed by pore membrane formation (Bierbaum and Sahl, 2009).

The type-B lantibiotic mersacidin and actagardine are also able to interact with lipid II, inhibiting peptidoglycan synthesis (Brotz *et al.*, 1997; Brotz *et al.*, 1998), though they do not induce membrane pore formation. Besides, it has also been shown that, contrary to nisin, mersacidin can discriminate lipid I from lipid II, indicating that GlcNAc, the only difference between those molecules, contains part of the mersacidin recognition motif (Brotz *et al.*, 1998; Hsu *et al.*, 2003). The mersacidin Glu residue present in its C-terminal was shown to play an essential role in its antibacterial activity (Figure 7, page 15). The substitution of this residue by an Ala resulted in good yields of the mutated peptide, which showed markedly reduced activity (Szekat *et al.*, 2003). Therefore, it was speculated that this residue (also present in actagardine) might form the binding site with its target lipid II molecule (Zimmermann and Jung, 1997; Brotz *et al.*, 1998).

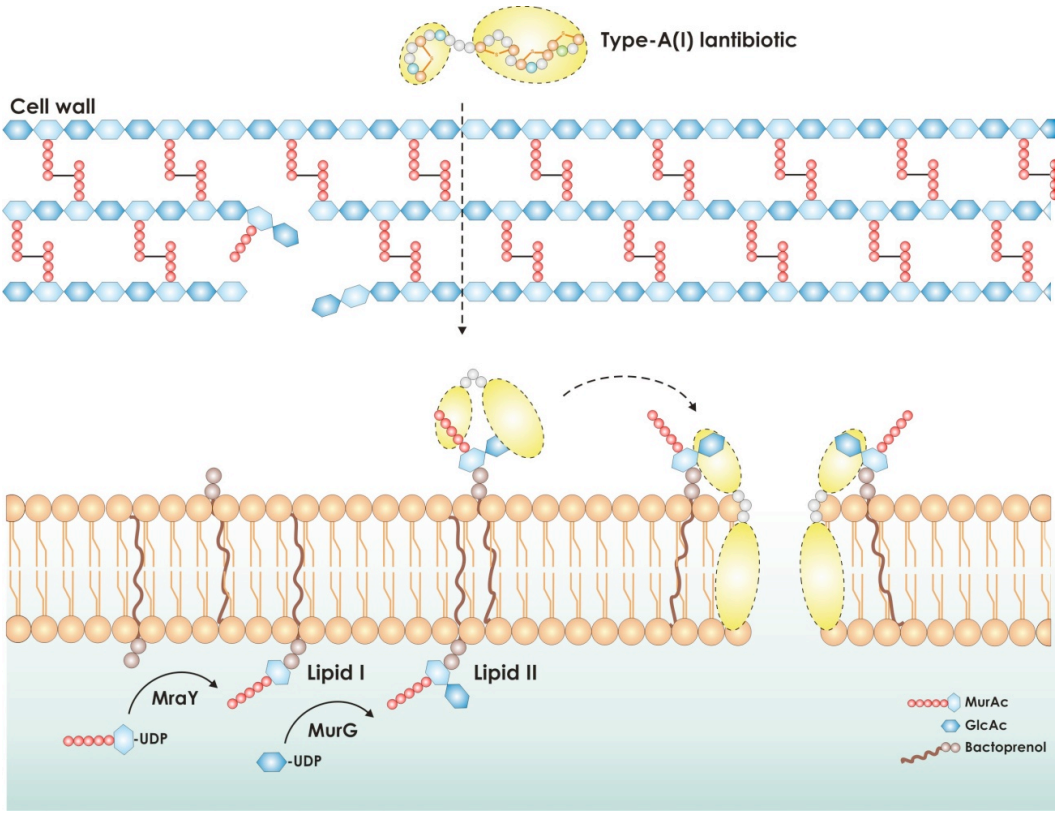


Figure 9: Schematic representation of the dual mode of action of type-A(I) lantibiotics e.g. nisin. The mechanism involves the binding of the lantibiotic to lipid II, preventing the correct cell wall synthesis. Furthermore, lipid II can be used as a docking molecule to initiate a process of membrane insertion and pore formation that leads to rapid cell death. Adapted from Cotter *et al.* (2005a).

The α-peptides of two-component lantibiotics are homologous to mersacidin, also possessing the Glu residue important for mersacidin activity in the C-terminally located TxS/TxEC conserved motif. The β-peptides seem to be more closely related to type-A lantibiotics. It was shown that the lacticin 3147 peptides, Ltnα and Ltnβ, could act synergistically and sequentially at a 1:1 molecular ratio (Morgan *et al.*, 2005). However, individually, the peptides do not show a high antagonistic effect. Further studies supported the model proposed by Wiedemann *et al.* (2006) for lacticin 3147 activity, which should involve three steps: first, the Ltnα peptide should bind to lipid II; this binding will induce or stabilize the conformation of Ltnα peptide that would facilitate the interaction with Ltnβ peptide and enable the formation of a complex involving both peptides and the lipid II molecule. After binding to the Ltnα:lipid II complex, the Ltnβ peptide will be able to adopt a transmembrane conformation, which would allow the formation of a pore in the membrane, leading to cell death (Wiedemann *et al.*, 2006). Therefore, lacticin 3147 antibacterial mode of action is likely to be equivalent to that of mersacidin, in lipid II binding and nisin, in the membrane pore formation. Despite some structural differences between the haloduracin and lacticin 3147 components, it is believed that they share the same mechanism of action (Oman and van der Donk, 2009b).

1.2.6 Self-protection of the producing strains

The biosynthetic gene clusters of lantibiotics often possess other ORFs apart from those already discussed, which have been associated to a self-protection mechanism of the producer strain against the harmful effects of its own product. The diverse self-protection systems described consist of: i) individual immunity proteins, generically termed as LanI; ii) an ABC transporter, usually comprising two or three subunits and termed LanFE(G) and iii) an accessory factor LanH, which most likely acts as an ancillary protein for the assembly of a functioning ABC transporter (Figure 10) (Draper *et al.*, 2008). The *lan*H gene is not present in the majority of the lantibiotic gene clusters known so far, contrary to *lanI* and *lanFE(G)* ORFs (Figure 3, page 9).

Regarding the LanI elements, a very low sequence similarity is found among these proteins. However, they are generally hydrophilic lipoproteins harboring a hydrophobic N-terminal domain, which can be involved in its anchoring to the cell membrane (Willey and van der Donk, 2007). The contribution of LanI protein for nisin self-protection was demonstrated when a nisin-sensitive strain was complemented with the plasmid encoding the NisI protein (together with NisABCT) and acquired very close immunity levels to those exhibited by the wild-type (Kuipers *et al.*, 1993a). Moreover it was shown that NisI was

shown to exist in two forms: a lipid-free protein present in the bacterial supernatant and a membrane associated lipoprotein. Both of these forms had the capability to interact by binding with nisin molecules. In possession of these evidences, it was proposed that NisI could aggregate with the lantibiotic, preventing pore formation and therefore protecting the bacterial cell (Figure 10) (Qiao *et al.*, 1995; Koponen *et al.*, 2004; Draper *et al.*, 2008). This contribution has also been proposed for the SpaI, PepI and CylI proteins in the self-protection mechanisms of subtilin, pep5 lantibiotic and the two-component lantibiotic cytolysin, respectively (Coburn *et al.*, 1999; Hoffmann *et al.*, 2004; Stein *et al.*, 2005). Nevertheless, and contrary to several other lantibiotics, PepI and CylI were the only determinants associated with the self-protection mechanisms of their producing strains.

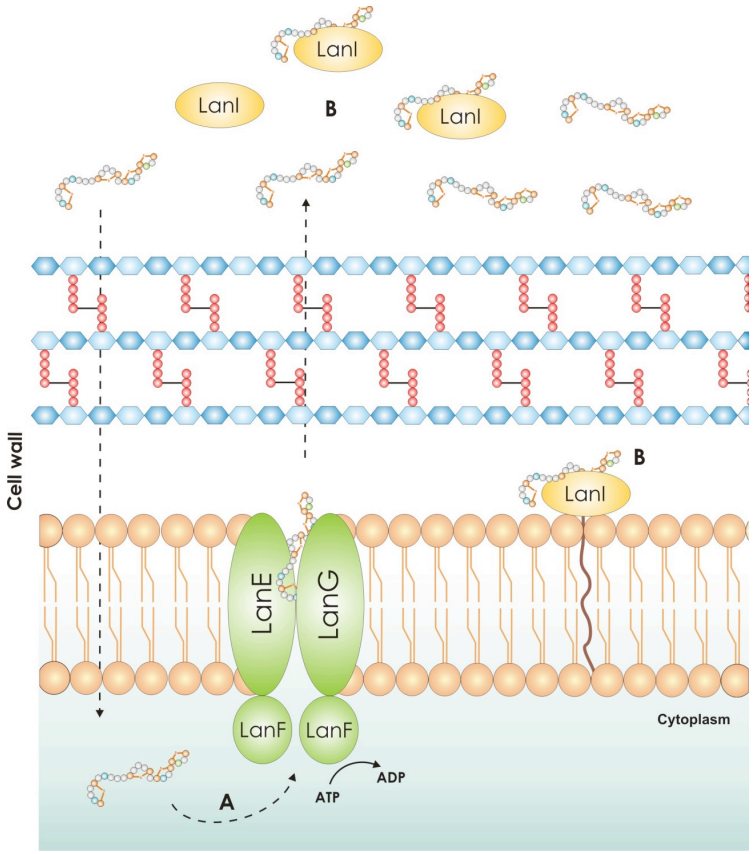


Figure 10: Structural organization of LanFEG and LanI peptides within the membrane of the lantibiotic producer. Two LanF peptides should combine either with two LanE peptides or, as represented above, with one LanE and a LanG peptide to form a functional ABC transporter. LanF functions as an ATPase, while LanE(G) forms the membrane spanning domain of the transporter (A). The LanI peptide can be found attached to the membrane via a lipid moiety or free in the supernatant as observed with NisI (B). Adapted from Draper *et al.* (2008).

Additionally or cooperatively to LanI, lantibiotic self-protection mechanism often imply the action of LanF, LanE and/or LanG. These proteins are individual domains of an ABC transporter (not related with the previously described LanT transporters). Therefore, the functionality of this ABC transporter is dependent on the combination of the LanF ATPase subunit with the two integral membrane peptides LanE and/or LanG (McAuliffe *et al.*, 2001b). Considering the nisin system, the disruption of each of the genes encoding LanFEG

proteins had no effect in the biosynthesis of the lantibiotic. However, these mutants showed an increased sensitivity when incubated with nisin, demonstrating the implication of these proteins in the *L. lactis* self-protection mechanism (Siegers and Entian, 1995). Thus, it has been suggested that NisFEG functions by excreting the nisin molecules that, once in the extracellular environment, were able to reenter to the producer cell (Stein *et al.*, 2003; Draper *et al.*, 2008). Either by cooperative or additive effect (still under debate), the NisFEG system, together with NisI is essential to achieve the full nisin-immunity phenotype of the producer strain (Ra *et al.*, 1999; Stein *et al.*, 2003; Takala *et al.*, 2004; Takala and Saris, 2006). The subtilin, epidermin and nukacin ISK-1 self-protection mechanism also resembles that of nisin regarding the ABC transporter functionality (Peschel and Gotz, 1996; Aso *et al.*, 2004b; Stein *et al.*, 2005). Even so, contrary to subtilin, in the epidermin and nukacin ISK-1 systems a candidate for the LanI protein could not be identified. However, another peptide also associated with epidermin and nukacin ISK-1 immunity was recognized, which is commonly designated by LanH. These molecules have been characterized as accessory factors acting as ancillary peptides for the assembly of the functional immunity ABC transporter LanFE(G) (Draper *et al.*, 2008). The studies involving LanH proteins are limited, however it was found that the NukH protein is able to interact with the nukacin ISK-1 lantibiotic, however no correlation between the immunity level and the NukH binding activity to the lantibiotic was found (Okuda *et al.*, 2005).

The mersacidin self-protection mechanism consists only of the MrsFEG ABC transporter system, which functionality should resemble that of the nisin transporter NisFEG. Moreover, it has been proposed that because mersacidin is incapable of pore formation, a sophisticated immunity mechanism is not required (Draper *et al.*, 2008).

The immunity peptides of two-component lantibiotics are interesting since it is not apparent whether they protect the cell against one particular peptide or both (Draper *et al.*, 2008). The immunity determinants encoded in the biosynthetic cluster of the two-component lantibiotic lacticin 3147 include the LctI and LctFE proteins, and both systems greatly contribute to the producer strain self-protection (Draper *et al.*, 2009). It was demonstrated that LtnFE has a higher ability to protect the cells against lacticin 3147 (Ltnα and Ltnβ together) than LtnI. However, LtnI exceeded LtnFE in its ability to protect the cell from the action of Ltnβ and they provided an equal level of protection to Ltnα (Draper *et al.*, 2009). In these systems where the LanG peptide is absent it is believed that the assembly of the active ABC transporter involves two LtnE and two LanF molecules.

Despite the similarly of structures among several lantibiotics, cross-immunity between lantibiotic producers is a rare event. Nevertheless, it was shown that PepI provided immunity to epicidin 280 (Heidrich *et al.*, 1998); NukH could interact with lacticin 481

(Heidrich *et al.*, 1998; 2005) and producers of the two-component lantibiotic staphylococcin C55 were shown to be cross-immune to lacticin 3147 (Draper *et al.*, 2008).

1.3 Nonribosomal antibiotics – a general overview

Some bacteria also produce the so-called conventional/traditional peptide antibiotics, which are not considered bacteriocins. An important criterion for such distinction relies on their distinct biosynthetic pathway, since the first are produced by multi-enzyme complexes (Nes *et al.*, 2007). These peptide antibiotics are synthesized via a template-directed, nucleic acid-independent ribosomal mechanism (Figure 11) (Caboche *et al.*, 2008; Felnagle *et al.*, 2008), which uses large multifunctional enzymes, referred as nonribosomal peptide synthetases (NRPSs) (Cane *et al.*, 1998). Although not recognized at the time, Alexander Fleming's discovery of penicillin in the late1920s introduced the world to the NRPS era, once the first step of penicillin biosynthesis involves the ACV-tripeptide production by a NRPS pathway (Smith *et al.*, 1990; Felnagle *et al.*, 2008).

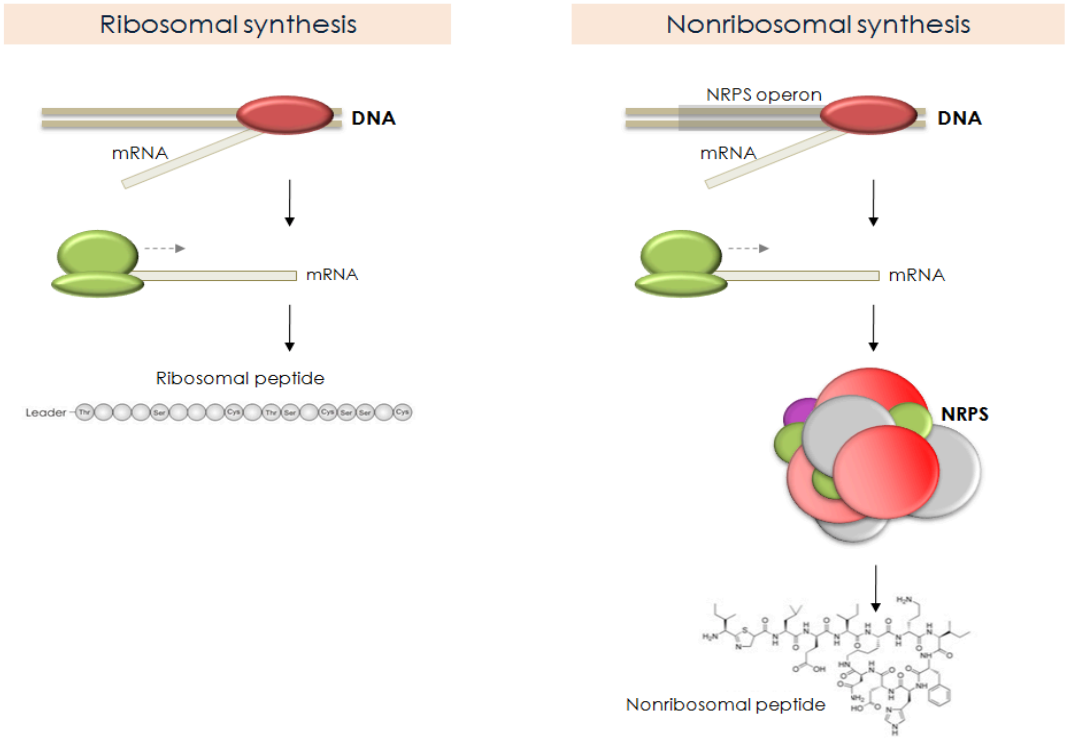


Figure 11: Schematic representation of the ribosomal and nonribosomal biosynthetic pathways involved in the production of bacteriocins and traditional antibiotics, respectively.

NRPS enzymes are organized in modules that are responsible for the catalysis of polypeptide chain elongation and possible associated functional group modifications. Each module can be dissected into different enzymatic units, which perform the necessary steps leading to the production of the final peptide (Figure 11). Therefore, at least three different domains can be identified in each NRPS module: i) the adenylation (A) domain, which selects the cognate amino acid and activates it as an amino acyl adenylate, ii) the peptidyl carrier protein (PCP) domain, which accepts the activated amino acid and transports it through the NRPS catalytic centers iii) the condensation (C) domain that catalyzes the formation of the peptide bond (Figure 12) (Finking and Marahiel, 2004). Moreover, each module can also accommodate additional domains generally responsible for some of the unique characteristics of nonribosomal peptides. Finally, the synthesis is terminated by the thioesterase (TE) domain, which is incorporated in the last NRPS module (Finking and Marahiel, 2004). All of these domains can be identified at the protein level by characteristic, highly conserved sequence motifs normally designated as core-motifs (Schwarzer *et al.*, 2003). The nonribosomally-produced peptides can undergo futher modifications such as glycosylation, which are introduced by associated enzymes. These enzymes are typically encoded in the same biosynthetic gene cluster of the NRPS enzyme and include glycosyl transferases, halogenases and hydroxylases (Walsh, 2004).

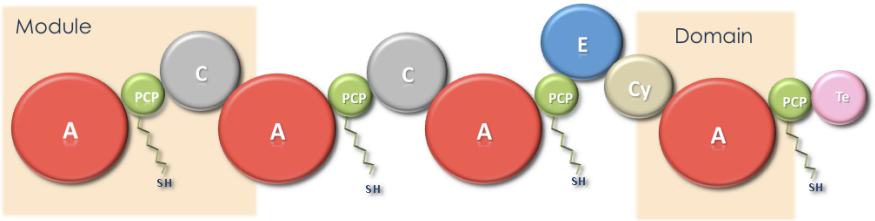
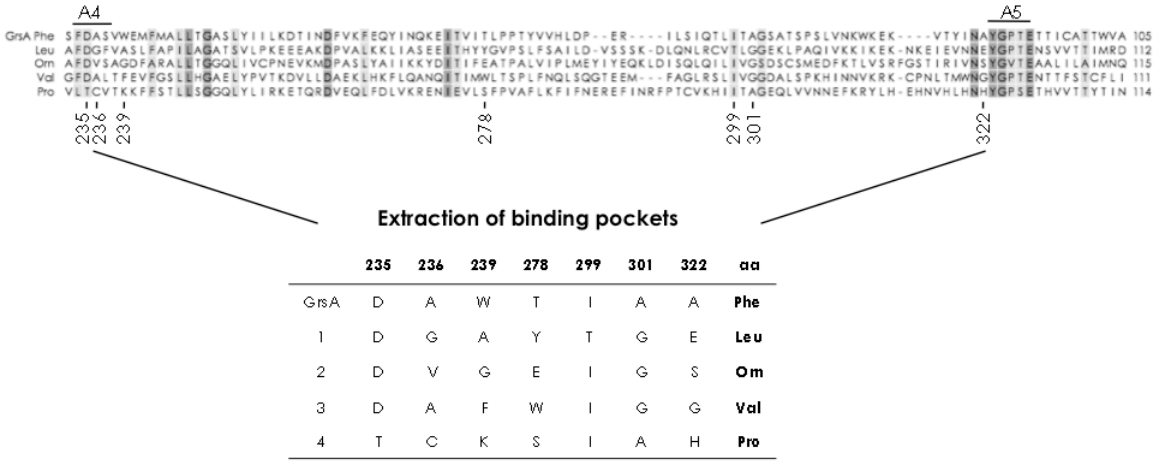


Figure 12: Schematic representation of the modular organization of a NRPS. Each module is responsible for the addition of an amino acid to the peptide chain. Thus, each domain exerts a specific activity (e.g. amino acid recognition and activation, transport among the catalytic centers and peptide bond formation) in the functionality of each module. The Te domain is only present in the termination module and is responsible for the release of the final peptide from the NRPS.

The A domain is probably the most important domain from each module, since it is responsible for the amino acid composition of the final peptide. Therefore, these have been classical candidates for the NRPS bioengineering prospects (von Dohren *et al.*, 1999). Based on the crystal structure of the phenylalanine activating A domain of the gramicidin NRPS (GrsA), Conti *et al.* (1997) the 10 residue positions that are crucial for its

substrate recognition were identified (Rausch *et al.*, 2005). The comparison of these residues with those of other A domains of already characterized peptides allowed the establishment of an association between these 10 residues and the specificity of the amino acid activated. This constituted the so-called nonribosomal code, where the residues of the substrate binding pocket constitute the codons of the nonribosomal synthesis (Schwarzer *et al.*, 2003). The nonribosomal code was firstly established by Stachelhaus *et al*. (1999) and by Challis *et al*. (2000) and it has been constantly enlarged and updated since then. The specificity-conferring amino acids can be extracted from the primary sequence of the A domains between the two core-motifs (A4 and A5) (Stachelhaus *et al.*, 1999; Challis *et al.*, 2000). With the application of this nonribosomal code, the substrate specificity has been predicted with fairly high accuracy (Figure 13) (Schwarzer *et al.*, 2003).



	235	236	239	278	299	301	322	aa
GrsA	D	A	W	T	I	A	A	**Phe**
1	D	G	A	Y	T	G	E	**Leu**
2	D	V	G	E	I	G	S	**Om**
3	D	A	F	W	I	G	G	**Val**
4	T	C	K	S	I	A	H	**Pro**

Figure 13: Representation of the binding pockets extraction methodology involved in the prediction of A domain specificity according to Stachelhaus *et al.* (1999). The reference sequence for the amino acid alignment should be the Phe-activating domain of the gramicidin GrsA NRPS. The sequence deduced from the amino acids located in the defined positions, constitutes the so-called nonribosomal code.

In bacterial systems, genes coding for NRPSs enzymes are usually organized in operons that can span regions of 6-45 Kb. A good example of that is the gene cluster of bacitracin A NRPSs. This enzyme is encoded in an operon of 42.1 Kb and synthesizes this well-known 12 amino acid antibiotic (Figure 14)(Konz *et al.*, 1997).

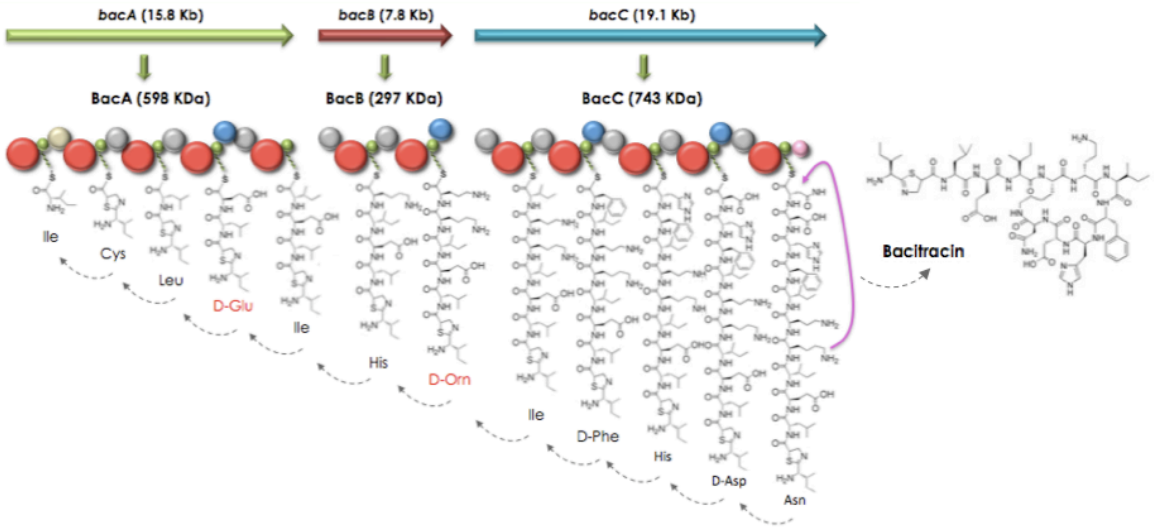


Figure 14: Schematic representation of the operon involved in bacitracin A production by the Bac NRPS. A total of twelve modules distributed between three NRPSs (BacA, BacB and BacC) are involved in the process of peptide chain elongation along the protein template. Adapted from Schwarzer *et al.* (2003).

Thus, NRPSs are used simultaneously as template (because the amino acid to be incorporated is determined by the module) and biosynthetic machinery (it is the module that harbors all the necessary catalytic functions) (Cane *et al.*, 1998; Finking and Marahiel, 2004). The modular organization of NRPSs offers the possibility of artificial alterations of the protein template, reprogramming it for the synthesis of novel peptides. Using this approach, several modifications of these enzymes have been made, including i) replacing domains with different specificity, ii) deleting or inserting domains and iii) deleting or inserting entire modules, where the expected peptide has often been obtained (Menzella and Reeves, 2007). Nevertheless, combinatorial biosynthesis is still in development, but it is hoped that it could lead to the development of new bioactive compounds (Menzella and Reeves, 2007).

Research in the natural products field, including peptide antibiotics has significantly declined during the last decade(s). However, it is still believed that unknown NRPSs can constitute one of the most promising sources of new classes of bioactive compounds (Singh and Pelaez, 2008). Two main efforts have been undertaken in order to identify some of these compounds: i) introduction of metagenomic techniques, that can reveal new NRPS gene clusters directly from culturable and unculturable microorganisms in the natural environments (Li and Qin, 2005) and the ii) screening for cultivable organisms from extreme or unusual habitats (marine, sediments, deep sea, etc.), since it is expected that these microbial communities will have unique adaptations to their habitats, which might be

linked, in some cases, with the synthesis of unusual antibacterial compounds (Pathom-aree *et al.*, 2006; Zhao *et al.*, 2008). Some studies in these fields have already proven their utility for such purposes (Courtois *et al.*, 2003; Moffitt and Neilan, 2003; Schirmer *et al.*, 2005; Zhao *et al.*, 2008).

The conventional peptide antibiotics tend to have a broader range of activity, when compared with the bacteriocins, which are generally active against Gram-positive but not Gram-negative bacteria (Nes *et al.*, 2007; Sang and Blecha, 2008). The mechanism of action behind the antibacterial activity of the traditional peptide antibiotics highly depends on the compound family. The glycopeptides antibiotics such as vancomycin inhibit the transglycosylation (chain elongation) and transpeptidation (cross-linking) steps in the cell-wall assembly, through their binding to the C-terminal D-Ala–D-Ala peptide motif of the UDP-muramyl pentapeptide that is a precursor of the bacterial cell wall biosynthesis (UDP=uridine diphosphate) (Süssmuth, 2002). Another nonribosomal peptide antibiotic that interfere with the bacterial cell wall biosynthesis is bacitracin. Bacitracin prevents the dephosphorylation of undecaprenyl pyrophosphate (UPP) into undecaprenyl phosphate (UP) (Stone and Strominger, 1971), preventing the translocation of peptidoglycan precursors to the external side of the membrane (Bernard *et al.*, 2005). The anti-Gram-negative polymyxin B and colistin (polymyxin E) are pentacationic cyclic lipodecapeptides, which permeabilize the Gram-negative outer membrane and disrupt the cytoplasmic membrane. However, due to these characteristics they also tend to be cytotoxic, which has limited their application in clinical practice (Hancock and Chapple, 1999; Vaara, 2010). Other antibiotic peptides of nonribosomal origin, the streptogramins, act at the level of inhibition of translation through binding to the bacterial ribosome (Johnston *et al.*, 2002).

1.4 Objectives of this thesis

The work developed in this thesis falls within the scope of the search and characterization of novel antimicrobial compounds. In this context a *Bacillus licheniformis* strain (I89) was isolated and identified as a producer of antibacterial compounds. One of those compounds was partially characterized. In the present thesis further studies were performed in order to clarify the following aspects:

- Identify the diversity of antibacterial compounds produced by *B. licheniformis* I89.
- Obtain insights on the structure of some of these antibacterial compounds.
- Characterize, whenever possible, the biosynthetic clusters involved in their production.
- Understand the biosynthetic pathways involved in the production of some of the major antibacterial compounds.

Moreover, the diversity of potential novel nonribosomal synthetases (which produce a wide variety of antimicrobial peptides) was investigated through culture-independent techniques in some Portuguese lagoons and caves environments.

The strategies employed and the main results obtained during the development of this study are described in the following chapters:

Chapter II: the growth and antibacterial activity of *B. licheniformis* I89 were investigated. Also, the antibacterial compounds present on its supernatant were separated and their molecular masses determined.

Chapter III: the morphological variants of *B. licheniformis* I89 were characterized and related with the production of the antibacterial lantibiotic lichenicidin.

Chapter IV: the production of lichenicidin lantibiotic by *E. coli* is described. Using a heterologous expression system, the function of the ORFs involved in the lichenicidin biosynthetic pathway was exploited through the generation of several knockout mutants. Moreover, the structure-activity relationship studies were performed for each of the lichenicidin peptides.

Chapter V: the involvement of lichenicidin putative immunity determinants in the self-protection of the *E. coli* lichenicidin producer as well as in the rate of production was investigated.

Chapter VI: the ability of the lichenicidin machinery to participate in the production of other lantibiotics in *E. coli* was investigated.

Chapter VII: the screening of adenylation domains from water and soil samples collected from Portuguese lagoons and caves that can potentially be involved in the production of nonribosomal peptides was performed by culture-independent methods.

CHAPTER II

Antibacterial compounds produced by *Bacillus licheniformis I89*

2.1 Introduction

Bacillus licheniformis is a Gram-positive endospore forming and facultative anaerobe bacteria which belongs to the *B. subtilis* group (group II) of the genus *Bacillus* (Veith *et al.*, 2004). It is widely distributed as a saprophytic organism in the environment (Rey *et al.*, 2004). *B. licheniformis* strains produce several compounds with important commercial and agricultural applications. Thus, this specie is widely used in the fermentation industry of amylases, proteases and antibiotics (Rey *et al.*, 2004).

Regarding the nonribosomal antibacterials produced by *B. licheniformis*, bacitracin was the first compound of this class to be purified from this specie. At that time, it was found that bacitracin exhibited i*n vitro* activity against Gram-positive organisms and a few Gram-negative (gonococci and meningococci). It was also active *in vivo* against experimentally produced hemolytic streptococcal infections in mice and gas gangrene infections in guinea pigs (Johnson *et al.*, 1945). Nowadays, bacitracin is widely used for medical (topical applications) and veterinary purposes (He *et al.*, 2006). However, the production of bacitracin is dependent on the bacterial strain. In fact, the presence of the nonribosomal peptide synthetase (NRPS) responsible for the bacitracin production has been defined as a criterion to distinguish between different sub-groups of *B. licheniformis* strains (Manachini *et al.*, 1998; Ishihara *et al.*, 2002). Another well-known group of nonribosomally-synthesized lipopeptides produced by *B. licheniformis* strains and also exhibiting antibacterial activity are the lichenysins (Yakimov *et al.*, 1995). Lichenysin A is the best characterized of these compounds. Due to its toxicity, lichenysins are not commercialized as antibacterial compound (Mikkola *et al.*, 2000). However, these peptides represent one of the most powerful biosurfactants known so far. Therefore, an increased interest has been directed to such compounds, since surfactants are of major interest for a variety of applications, particularly the microbial enhanced oil recovery

(Yakimov *et al.*, 1998).

Table 2: List of antagonistic compounds produced by *B. licheniformis* strains, with reference to their temperature of production and molecular masses, when determined. [a)] Antibacterial compound produced anaerobically. [b)] Antibacterial compound heterologously expressed in *E. coli*. The molecular masses measured by MS analysis were represented with *. ND stands for information not available.

Strain	Source	Temperature of production	Antibacterial compound(s)	Molecular weight (Da)	References
A12	Natural cave	28 °C	A12-C	ND	(Gálvez *et al.*, 1993a)
			A12-A and A12-B	1430	(Gálvez *et al.*, 1993b)
26L-10/3RA	Buffalo rumen	39 °C	Lichenin[a)]	1400	(Pattnaik *et al.*, 2001)
490/5	Dairy food	55 °C	Bacillocin 490	2000	(Martirani *et al.*, 2002)
P40	Intestinal contents of Leporinus sp.	26 °C – 37 °C	Bacillocin P40	ND	(Cladera-Olivera *et al.*, 2004b; Cladera-Olivera *et al.*, 2004a)
A89	Hot spring	50 °C	A89	3249.7*	(Mendo *et al.*, 2004)
ZJU12	Soil	30 °C	ZJU12	3000	(He *et al.*, 2006)
SAFN031	Seaweed	28 °C	YbdN protein	30700	(Jamal *et al.*, 2006)
AnBa9	Slaughterhouse sewage waste sediments	37 °C	BhlA[b)]	8200*	(Thangamani *et al.*, 2009)
MKU3	Slaughterhouse sediments	30 °C	MKU3	<10000	(Kayalvizhi and Gunasekaran, 2010)

In the last twenty-years, *B. licheniformis* strains have also been described as producers of antagonistic compounds, other than those nonribosomally-synthesized (Table 2). Those compounds were identified as secondary metabolites of *B. licheniformis* strains isolated from several distinct environments including natural caves, dairy food, fish intestines and buffalo rumen (Gálvez *et al.*, 1993a; Pattnaik *et al.*, 2001; Martirani *et al.*, 2002; Cladera-Olivera *et al.*, 2004b). The majority of these substances are produced aerobically in a wide variety of temperatures ranging from 26 °C to 55 °C. Despite that, lichenin peptide was found to be synthesized exclusively in the absence of oxygen (Pattnaik *et al.*, 2001). The characterization of these compounds was especially focused on their chemical stability together with their antibacterial spectrum of activity (Table 3). Some of these studies were performed with extracts containing an unknown mixture of substances, rather than purified compounds. Regarding the spectrum of antibacterial activity, it was normally associated with Gram-positive bacteria, including clinically relevant specimens such as methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant *Enterococcus* (VRE). Still, the compounds produced by some strains like *B. licheniformis* P40 and ZJU12 were also able to inhibit the growth of Gram-negative isolates including *Aeromonas* sp., *Enterobacter aerogenes, Erwinia carotorovora, Pasteurella haemolytica* and *Xanthomonas* oryzae (Cladera-Olivera *et al.*, 2004b; Cladera-Olivera *et al.*, 2004a; He *et al.*, 2006).

Table 3: Characterization of antimicrobial compounds (not including those nonribosomally-synthesized) produced by *B. licheniformis* strains considering their spectrum of activity and stability to the processing of several proteases. NT stands for not tested.

Antimicribial compound(s)	Antibacterial activity		Stability of activity		References
	Active	Inactive	Sensitive	Insensitive	
A12-A and A12-B	*Naeglena fowlen, Criptococcus neoformans, C. glutamicum, Sarcina* sp.	*Acanthamoeba sp., M. mucedo, Sporothrix schenchii*		Trypsin Pronase Proteinase K Carboxypeptidase A	(Gálvez *et al.*, 1993b)
A12-C	*Microsporum canis, Mucor mucedo, Trichophyton mentagrophytes, C. glutamicum, Sarcina sp.*	*Candida albicans, Bacillus cereus, Bacillus circulans, Baciullus laterosporus, Bacillus licheniformis, Bacillus subtilis, Enterococcus faecalis, Enterococcus faecium, Enterococcus durans, Micrococcus luteus, Planococcus citreus, Staphylococcus aureus, Alcaligenes faecalis, Enterobacter cloacae, Escherichia coli, Klebsiella pneumoniae, Proteus sp., Pseudomonas reptiivora, Salmonella typhimurium* *Cryptococcus neoformans, Penicillium notatum, Aspergillus niger*			(Gálvez *et al.*, 1993a)
Lichenin[a)]	*Eubacterium ruminantium, Streptococcus bovis, Ruminococcus favefaciens, Ruminococcus albus*	*B. cereus, Staphylococcus aureus, Bacteroides amylophilus, E. coli, Pseudomonas aeruginosa, S typhimurium, Selenomonas ruminantium*	Proteinase K Pronase E α-chymotrypsin	Trypsin	(Pattnaik *et al.*, 2001)
Bacillocin 490	*Bacillus anthracis, B. licheniformis, Bacillus stearothermophilus, Bacillus smithii, B. subtilis, Listeria innocua, Staphylococcus epidermidis*	*Bacillus thuringensis,S. aureus, Streptococcus thermophilus, E. coli, Enterococcus faecalis, P. aeruginosa, Proteus mirabilis*	Proteinase K Pronase E	Trypsin Chymotrypsin	(Martirani *et al.*, 2002)
Bacillocin P40	*B. cereus, B. subtilis, Corynebacterium fimi, Lactobacillus acidophilus, Listeria monocytogenes, Listeria innocua, Rhodococcus sp., Streptococcus sp., Aeromonas sp., Enterobacter aerogenes, Erwinia carotorovora, Pasteurella haemolytica*	*Actinomyces sp., S. aureus, S. epidermidis, E. coli, Pseudomonas sp., Salmonella enteritidis*	Pronase E Butanol TCA	Trypsin Papain Proteinase K	(Cladera-Olivera *et al.*, 2004b; Cladera-Olivera *et al.*, 2004a)
A89	*B. subtilis, S. aureus, M. luteus*	*C. albicans, Saccharomyces cerevisae E. coli, P. aeruginosa*		Trypsin α-chymotrypsin Cardosin A and B	(Mendo *et al.*, 2004)
ZJU12	*B. subtilis, E. faecium, Micrococcus flavus, S. aureus (including MRSA), S. epidermidis, Xanthomonas oryzae and fungi associated with plants*	*Acinectobacter baumanii, E. cloacae, E. coli, Proteus sp., P. aeruginosa*	Proteinase K Trypsin		(He *et al.*, 2006)
YbdN protein	*Enterococcus sp. (including VRE), L. monocytogenes, S. aureus (including MRSA)*		Proteinase K Pronase Trypsin		(Jamal *et al.*, 2006)
BhlA[b)]	*B. cereus, B. subtilis, Kurthia gibsonii, Lactobacillus acidophilus, M. luteus, Pediococcus acidilactici, S. aureus (including MRSA), S. epidermidis*	*L. innocula, L. seeligeri, P. aeruginosa, P. fluorescens*	NT	NT	(Thangamani *et al.*, 2009)
MKU3	*B. cereus, B. subtilis, B. smithii, E. faecalis, K. gibsonii, L. lactis, L. plantarum, L. acidophilus, L. fermentum, L. innocua, L. seeligeri, Leuconostoc mesenteroides, M. luteus, Pediococcus acidilactici, S aureus, S. epidermidis*	*E. coli, P. fluorescens, Serratia marsacens*		Trypsin Pronase K Proteinase K	(Kayalvizhi and Gunasekaran, 2010)

Other targets of the compounds produced by *B. licheniformis* ZJU12 strain include the plant associated fungi (He *et al.*, 2006). Also, the peptides A12A and A12B isolated from *B. licheniformis* A12 strain revealed anti-amoebicidal activity (Gálvez *et al.*, 1993b). Concerning the chemical stability of these compounds, it has been described that bioactivity can be affected by several proteases, although is highly dependent on the producer strain or on the compound.

B. licheniformis I89, object of the present study, was isolated from an hot spring environment in São Miguel, Azores, Portugal (Mendo *et al.*, 2000). Cell-free supernatants of this strain, after fermentations at 50 °C, were able to inhibit the growth of Gram-positive bacteria such as *B. subtilis*, *M. luteus* and *S. aureus* but not the Gram-negative *E. coli* and *Pseudomonas* spp. Also, activity against yeasts like *Saccharomyces cerevisiae* and *Candida albicans* was not detected. A peptide antibiotic referred as A89 and characterized by the molecular mass of 3249.7 Da was initially isolated from the strain's supernatant (Mendo *et al.*, 2004). Regarding the stability of A89 to proteases, it was found that the aspartic proteases cardosin A and B and the serine proteases trypsin and α-chymotrypsin had no influence on its bioactivity. Additionally It was also shown that its bioactivity was extremely stable to a wide range of pH conditions (3.5 and 8) (Mendo *et al.*, 2004).

One of the objectives of this dissertation was to investigate if the antagonistic activity exhibited by *B. licheniformis* I89 strain was exclusively due to the production of the A89 peptide or also to other compounds. Additionally, determination of A89 amino acid content was also one of the aims of the present study. For this purpose, pure A89 peptide was required. The characterization and comparison of the *B. licheniformis* I89 antagonistic activity produced at two distinct temperatures (37 °C and 50 °C). The results obtained are presented in this chapter. Moreover, a protocol for the separation of antibacterial compounds from the I89 cell-free supernatants was also developed.

2.2 Results

2.2.1 Growth and antibacterial activity of *B. licheniformis* I89

Previously, it was established that *B. licheniformis* I89 strain produces an antibacterial compound, designated by A89, when it is grown in medium M at 50 °C. However, the same study demonstrated that if A89 peptide was incubated at 50 °C prior to bioassay, a decrease of 10 % in its activity was verified when compared with the pre-incubation at 37 °C, suggesting that at this temperature A89 stability and/or activity is retained (Mendo *et al.*, 2004). In the present study, it was observed that *B. licheniformis* I89 colonies were

able to inhibit *M. luteus* growth when incubated together for at least 16 hours, on tryptic soy agar (TSA) plates at 37 ºC. Therefore, considering the results obtained, it was decided to characterize the growth, sporulation and antibacterial activity of *B. licheniformis* I89 also at 37 ºC and compare them with the previously tested temperature of 50 ºC (Figure 15 and Figure 16).

The bacterial growth was evaluated every four hours using two distinct indirect methodologies: i) measurement of the optical density and ii) viable cells counts on agar plates (Figure 15A/B). Analysis of the obtained curves showed that the growth of I89 strain could be characterized by a lag phase of approximately four hours, followed by an exponential phase of around eight hours at both the conditions tested. The calculated generation time during this period was approximately 115 and 125 minutes at 37 ºC and 50 ºC, respectively. The stationary phase plateau was reached after approximately twenty hours of incubation. Overall, the growth kinetics of *B. licheniformis* I89 was similar at both temperatures. Nevertheless, the bacterial density and consequently the number of viable cells were generally higher at 37 ºC than at 50 ºC.

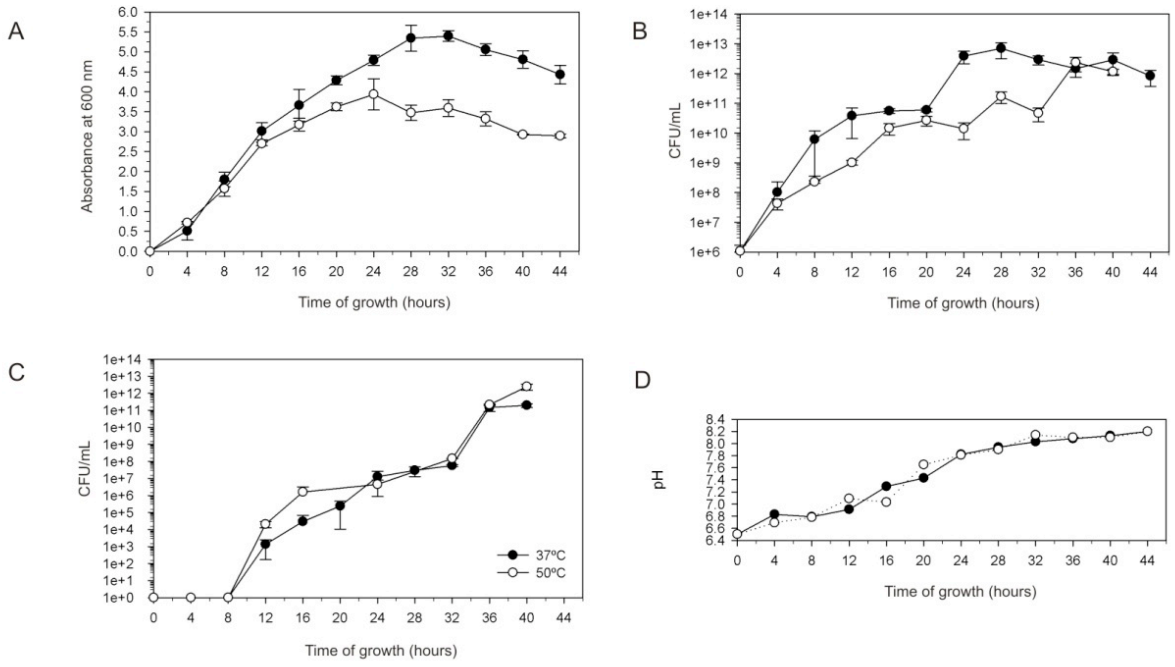


Figure 15: Growth parameters measured in batch cultures along the *B. licheniformis* I89 growth in medium M batch cultures, incubated at 37 ºC (black circles) or 50 ºC (white circles). Parameters analyzed included the optical density (A), determination of viable cells (B), production of spores (C) and variation of the supernatant pH (D).

Members of *Bacillus* spp. are characterized by the production of endospores that can remain dormant for extended periods of time. Therefore, in the present study the sporulation activity of I89 strain over time at both 37 °C and 50 °C was investigated (Figure 15C). The elimination of vegetative cells was performed by treatment at high temperature (80 °C) for 20 minutes. If present, the spores could survive to such conditions and allowed to germinate on fresh agar plates incubated at 37 °C or 50 °C. It was observed that I89 strain sporulation process initiated during the late exponential phase of growth at both of the temperatures tested. Along the stationary phase, the number of spores present in the cultures increased, reaching its plateau after 40 hours. Thus, differences on the sporulation rate between incubations at 37 °C and 50 °C were not perceptible.

Regarding the variation of the pH supernatant along the bacterial growth, it was found that pH values ranged from the initial pH 6.5 up to approximately pH 8.0 after 44 hours of growth in an almost perfect linear progression (Figure 15D). Moreover, this behavior was similar in both incubation temperatures.

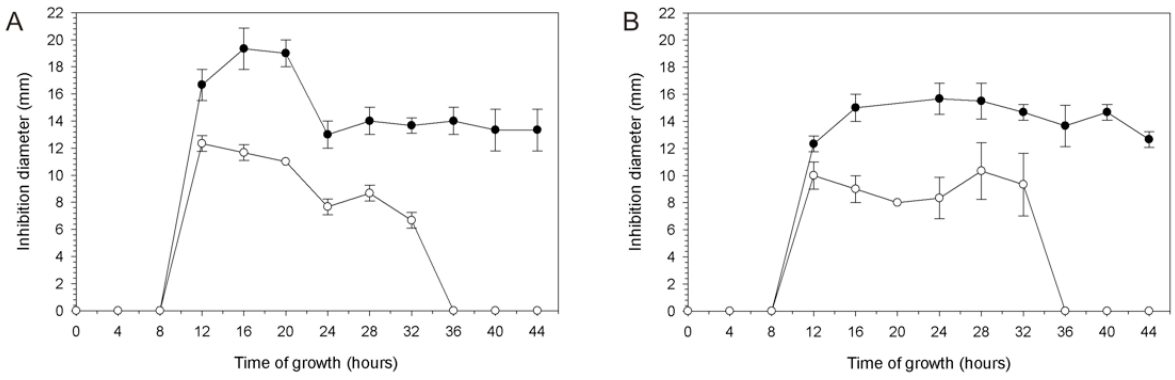


Figure 16: Antibacterial activity of *B. licheniformis* I89 cell-free supernatants (A) and cell wall washes (B) fractions when cultured at 37 °C (black circles) and 50 °C (white circles).

As previously referred, the antibacterial activity of *B. licheniformis* I89 was first investigated on its fermentation supernatants. Herein, we also evaluated the bioactivity of the compounds that remained associated with the bacterial cell wall through the analysis of cell wall associated fractions (Figure 16). It was showed that bioactivity seemed to be synchronized with the stationary phase at both temperatures either in the supernatants or the cell wall fractions. However, the inhibition zone diameters resulting from the fermentations at 37 °C were generally larger (> 2 mm) than those at 50 °C. Moreover, after 32 hours of incubation, the fractions removed from the cultures grown at 50 °C completely

lost their ability to inhibit *M. luteus* growth. That was not observed when the cultures were grown at 37 °C regardless of some decrease in the activity observed after 20 hours of incubation. The A89 antibacterial peptide was previously isolated from cell-free supernatants cultures of *B. licheniformis* I89 grown at 50 °C (Mendo *et al.*, 2004). In the present study it was observed that cell-free supernatant of cultures grown at 37 °C also have the ability to inhibit the growth of *M. luteus*. Thus, this fraction was submitted to LC-ESI-MS analysis to confirm if the A89 peptide was also produced at this temperature. The characteristic molecular mass of A89 peptide (M= 3250 Da) was successfully identified, proving that this peptide is also contributing for the bioactivity of the cultures grown at 37 °C.

2.2.2 Spectrum of antibacterial activity of *B. licheniformis* I89 supernatants

The antibacterial activity of *B. licheniformis* I89 supernatants against *M. luteus*, *S. aureus* and *B. subtilis* was previously reported by Mendo *et al.* (2004). Here, the antagonistic activity of *B. licheniformis* I89 supernatants resulting from cultures grown at 37 °C was tested against other Gram-positive and Gram-negative bacteria, including some clinical isolates (Table 4). The results showed that *Listeria monocytogenes*, *Enterococcus faecium*, *Enterococcus faecalis* and *Haemophilus influenzae* are also susceptible to the antibacterial produced by the I89 strain at 37 °C. Interestingly and considering the *S. aureus* isolates it was observed that the inhibition diameters obtained for the MRSA isolate was comparable to those observed for the two methicillin-sensitive strains.

Table 4: Susceptibility of Gram-positive and Gram-negative isolates to supernatant extracts of *B. licheniformis* I89 fermentations. Susceptibility values are presented as a ratio between the diameter of the tested strain and the more susceptible strain *M. luteus* ATCC 9341. MRSA stands for methicillin-resistant *Staphylococcus aureus*, whereas MSSA stands for methicillin-sensitive *Staphylococcus aureus*.

Strains	Susceptibility (%)	Observations
Gram-positive		
Enterococcus faecalis ATCC 29212	45	
Enterococcus faecium 547261	58	Clinical isolate
Haemophilus influenzae 121642	68	Clinical isolate
Listeria monocytogenes	71	
Micrococcus luteus ATCC 9341	100	
Staphylococcus aureus ATCC 29213	52	MSSA
Staphylococcus aureus 547263	55	Clinical isolate; MRSA
Staphylococcus aureus 137109	55	Clinical isolate; MSSA
Gram-negative		
Escherichia coli ATCC 35218	0	
Klebsiella pneumoniae 100603	0	Clinical isolate
Pseudomonas aeruginosa In99	0	Clinical isolate

2.2.3 Separation of antibacterial compounds produced by *B. licheniformis* I89

In order to understand if other compounds in addition to the A89 peptide could be contributing for *B. licheniformis* I89 bioactivity, the n-butanol supernatants extracts (referred as supernatant extracts) resulting from fermentations at 37 ºC were separated through diverse analytical procedures and systematically tested for their antibacterial activity. The protocols used included solid phase extraction (SPE) and preparative high-pressure liquid chromatography (prepHPLC).

2.2.3.1 Solid phase extraction of crude extracts

The first step of this analysis involved the SPE of supernatant extracts. Briefly, after an incubation period of 16-18 hours the culture cell-free supernatants were extracted with n-butanol as previously described by Mendo *et al.* (2004). After the evaporation of the organic solvent, the extracts were dissolved and applied to a pre-equilibrated C8 SPE column. The compounds bound to the carbon matrix were sequentially washed with solvents of different polarities. In order to identify with which solvent the antibacterial compounds were eluted, each of the fractions was bioassayed against *M. luteus*. Inhibition zones were exclusively observed in those fractions resulting from the column wash with 90 % and 70 % of ACN:water solutions. LC-ESI-MS analysis of these fractions revealed the presence of two major compounds possessing slightly different retention times and molecular masses of 3020 Da and 3250 Da. Thus, the A89 peptide (M= 3250 Da) was present in these fractions and the 3020 Da peptide was designated as B89. Since the antibacterial activity was confined to these two fractions, several SPE procedures were performed and the collected 90 % and 70 % ACN:water washes were pooled together and concentrated. The components of this SPE-treated extract were further separated by prepHPLC.

2.2.3.2 prepHPLC of SPE-treated supernatant extracts

The first prepHPLC separation consisted of a 20 min gradient, where all the eluted fractions were collected, concentrated and bioassayed. The results showed that the compounds possessing antibacterial activity were eluted from the column along seven minutes, starting at approximately R_t= 7 min after the beginning of the prepHPLC run (Figure 17). The profile of the inhibition zones measured along this period suggested the presence of, at least, three different antibacterial compounds, represented by the fractions I, II and III (Figure 17). The vial exhibiting the major inhibition zone from each of these fractions was analyzed by LC-ESI-MS (Figure 18). The fraction I was characterized by

the presence of several compounds. Therefore, in the present study this fraction was not subject to further analysis. On the contrary, in fractions II and III a major peak with a retention time of R_t= 17.9 min and R_t= 18.33 min, respectively, was identified. The molecular mass identified on that peak of the fraction II corresponded to the A89 peptide, while in fraction III the B89 molecular mass was detected. In order to obtain highly pure peptides, the first prepHPLC separation process was repeated several times and the vials corresponding to fraction II were pooled together. The same approach was used for the vials corresponding to the fraction III. After solvent evaporation, each fraction was subjected to a second prepHPLC analysis.

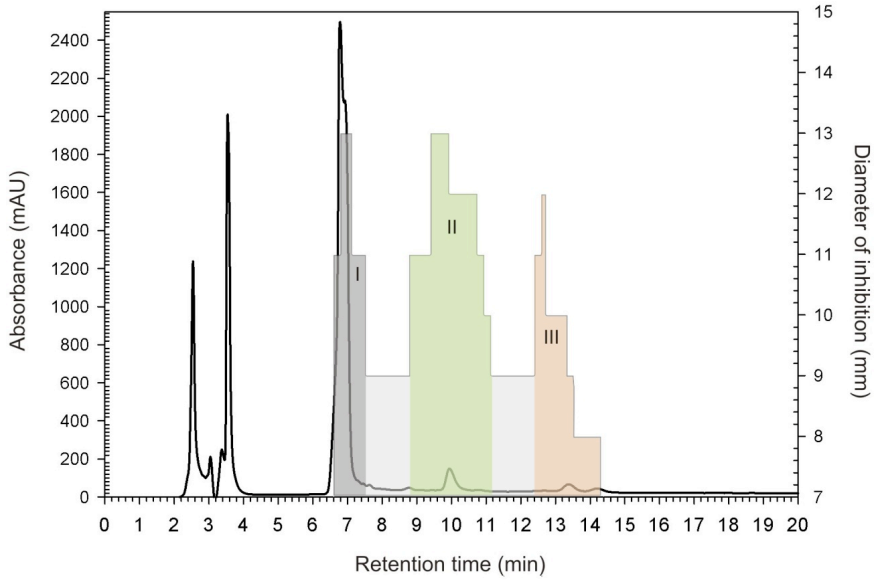


Figure 17: Chromatogram resulting from HPLC separation of SPE purified supernatant extracts from *B. licheniformis* I89 obtained at A= 210nm (black line). The bioactivity observed in each fraction is represented by dark grey (fraction I), green (fraction II) and orange (fraction III). No bioactivity was observed in the uncolored areas.

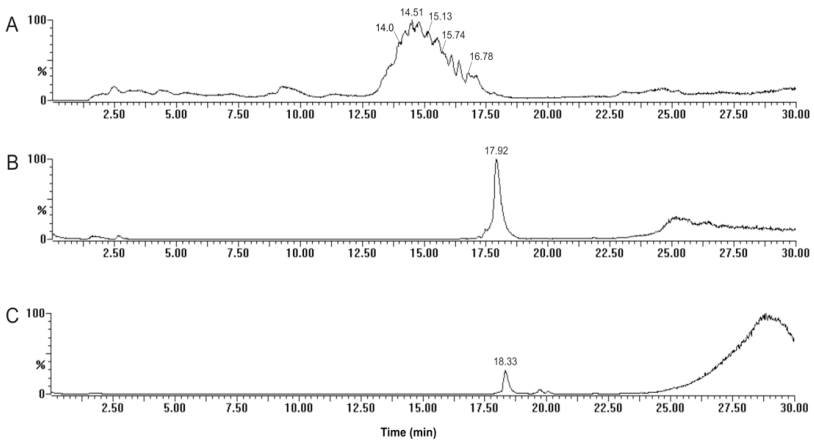


Figure 18: TIC chromatograms of the prepHPLC vials where the biggest inhibition zones were observed in the bioassay against *M. luteus* corresponding to fraction I (A; vial 21), fraction II (B, vial 29) and fraction III (C; vial 36) of Figure 17.

2.2.3.3 prepHPLC of fraction II

The fraction II was mainly composed by the A89 peptide. Nevertheless, its components were further separated by a prepHPLC procedure consisting of a 40 min gradient. Over this period, all the eluted fractions collected were concentrated and bioassayed against *M. luteus*. The presence of antibacterial activity was mainly detected in the vials containing compounds with retention times between seven and twelve minutes (Figure 19). Also, some bioactivity was identified on vials corresponding to a retention time of six minutes (Figure 19). According to the measured inhibition diameters, the fraction II was sub-divided into four sub-fractions that were further analyzed by LC-ESI-MS. The molecular mass of A89 was identified in all these sub-fractions. However, only the sub-fraction exhibiting the highest inhibition zone (sub-fraction 3) was exclusively constituted by this peptide. In the sub-fractions 1 and 2, apart from A89 peptide, compounds with molecular masses of 3284 Da and 3266 Da were also detected. The M= 3284 Da compound was the main constituent of sub-fraction 1, while sub-fraction 2 was primarily composed by a compound of M= 3266 Da. In the remaining sub-fraction 4, the most abundant peak corresponded to a M= 3278 Da. However, both A89 and B89 peptides were also detected in this fraction, although in a low abundance when compared with the M= 3278 Da compound. Therefore, the pure A89 peptide was obtained by pooling all the sub-fractions 3, resulting from several prepHPLC separations of fraction II. Finally, the Edman degradation was attempted on the A89 peptide. However, no results could be obtained probably due to the blockage of the reaction by the inaccessible N-termini.

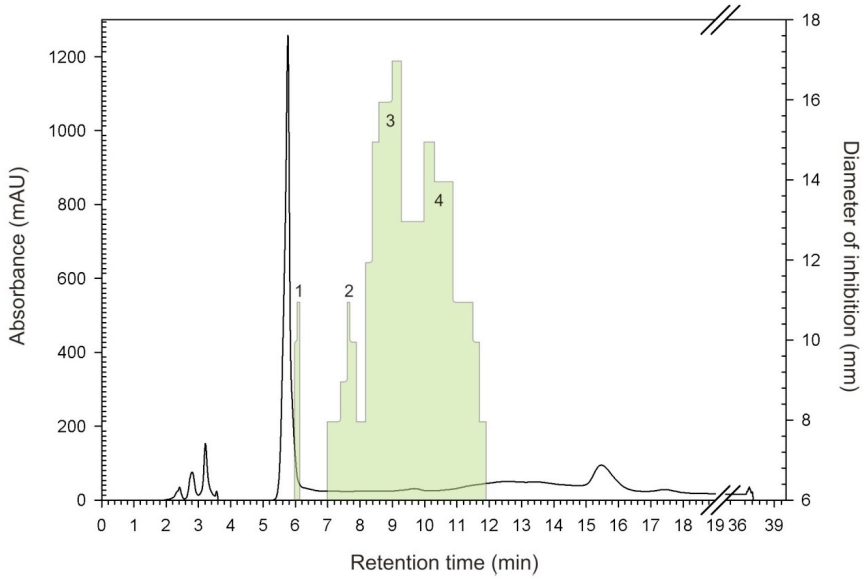


Figure 19: Chromatogram resulting from HPLC separation of fraction II at A= 210nm (dark black line), where the corresponding bioactivity is represented in green shade.

2.2.3.4 prepHPLC of fraction III

The fraction III was mainly composed by the B89 peptide. Even so its constituents were further separated involving the same prepHPLC procedure applied to the fraction II. The antagonistic activity observed along the applied gradient showed that antibacterial compounds were eluted in five distinct moments, which were designated as sub-fractions 1-5 (Figure 20). The biggest inhibition zone was detected for the first sub-fraction. LC-ESI-MS analysis of this fraction revealed the presence of the A89 peptide. On the remaining sub-fractions and also by LC-ESI-MS analysis, only the B89 peptide (sub-fractions 3 and 4) or a mixture of the B89 peptide with a compound possessing the molecular mass of 3038 Da (sub-fractions 2 and 5) could be detected. These compounds possess a difference in terms of molecular mass of +18 Da when compared with B89 peptide, suggesting that they compounds could be related with the B89 peptide, where a dehydration reaction did not occur. Therefore, the pure B89 peptide was finally obtained by mixing all the sub-fractions 3 and 4 resulting from several HPLC separations of fraction III. Finally, the B89 was also submitted to sequencing by Edman degradation. Similarly to A89, no results could be obtained.

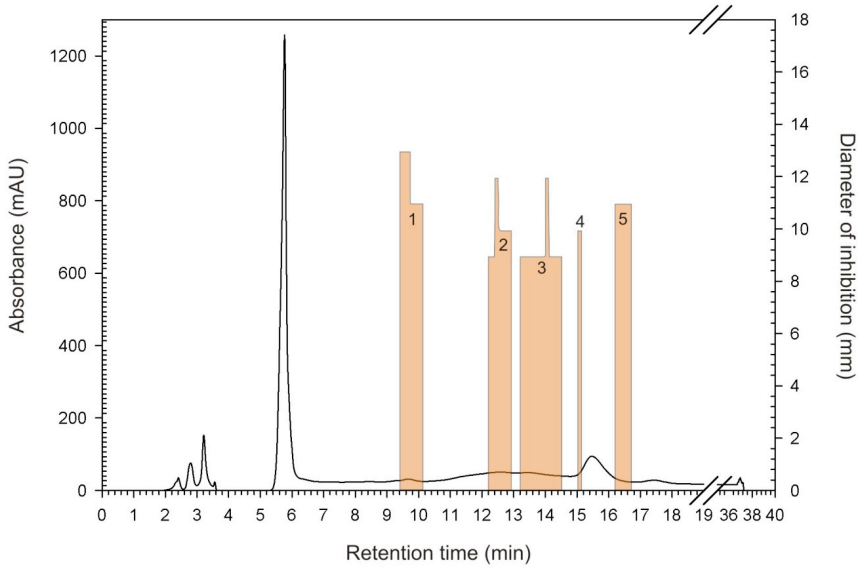


Figure 20: Chromatogram resulting from HPLC separation of fraction III at A= 210nm (dark black line), where the corresponding bioactivity is represented in orange shade.

2.2.4 Production of A89 and B89 peptides by *B. licheniformis* I89 at different temperatures

It was previously established that the A89 peptide was produced by *B. licheniformis* I89 in fermentations performed at 50 °C. As described in the previous section, the same

peptide can also be detected, together with another antibacterial peptide designated as B89, in the supernatant of I89 cultures grown at 37 °C. However it was not established if this newly identified peptide was also produced at 50 °C. Hence, the supernatants of *B. licheniformis* I89 grown at 37 °C and 50 °C were analyzed by LC-ESI-MS (Figure 21 and Figure 22). The resultant TIC chromatogram showed the presence of a mixture of compounds. However, the mass corresponding to A89 peptide was successfully identified in both samples after an elution time of approximately R_t= 18 minutes. In addition, the B89 peptide was also detected in both supernatants a few seconds later. The detailed analysis of the B89 TIC peak also revealed that the 3038 Da peptide was also present in both of the analyzed supernatants, demonstrating that its production also occurs at 50 °C (Figure 23).

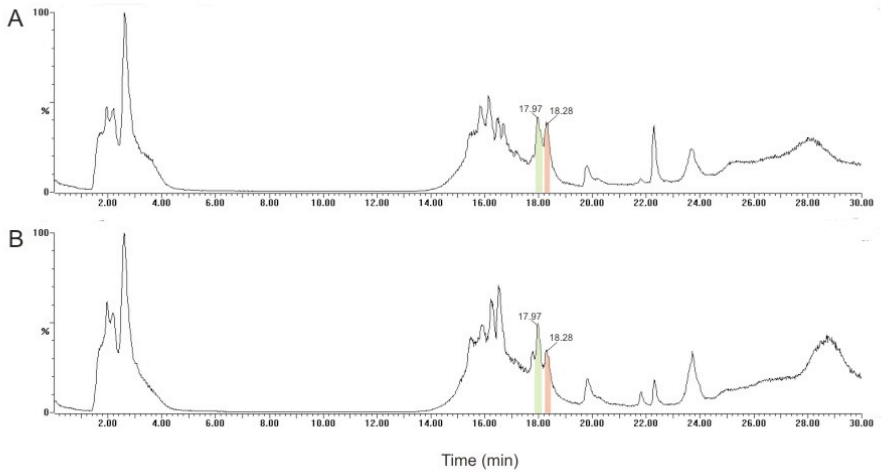


Figure 21: TIC chromatogram obtained from LC-ESI-MS analysis of supernatant butanol extracts from *B. licheniformis* I89 fermentations at 37 °C (A) and 50 °C (B). The green area corresponds to the peak where A89 the molecular mass was detected and the orange area where B89 molecular mass was identified.

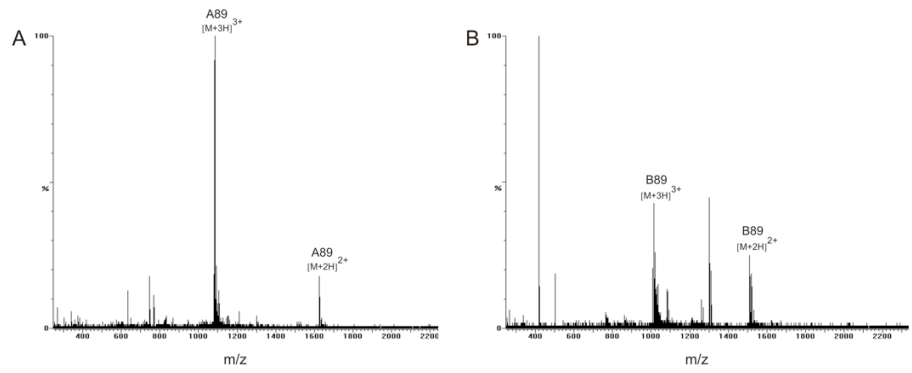


Figure 22: Mass spectra corresponding to the analysis of the two peaks highlighted in the **Figure 21**, where the A89 (A) and B89 (B) characteristic molecular masses could be identified. The double and triple charged ions of both peptides were identified in conjugation with some ions corresponding to fragments of each peptide.

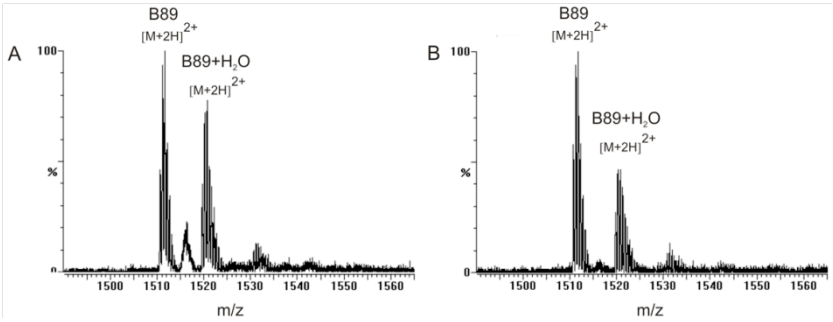


Figure 23: Detail of the double charged ions identified in the peak corresponding to a R_t= 18.20 min. The figure shows that the B89 peptide and a peptide with an additional water molecule are produced at both 37 ºC (A) and 50 ºC (B).

2.2.5 Nonribosomal adenylation domains present in the *B. licheniformis* I89 genome

The bacitracin and lichenysin nonribosomal peptide synthetases (NRPSs) have been detected in several *B. licheniformis* strains. Nevertheless, in the present study, the molecular masses of these two antibacterials were never detected in the supernatants or cell wall associated fractions from *B. licheniformis* I89 cultures. In order to investigate if I89 strain possessed NRPSs that could be involved in the biosynthesis of the A89 or B89 peptide, a library of its NRPS adenylation (A) domains was constructed. In total, only five different A domains were identified and designated as MT11, MT12, MT15, MT28 and MT75. The comparison of the amino acid sequence of these fragments with the genomes of *B. licheniformis* ATCC 14580 and *B. licheniformis* DSM13 was performed through the web-based BLAST software. The results showed that MT11, MT12, MT15 and MT28 possessed high homology with A domains belonging to the three sub-units of the lichenysin NRPS as represented in Figure 24. Regarding the MT75 sequence, it was found that its closely related A domain belongs to a putative and uncharacterized siderophore NRPS (Figure 24). Therefore, no potential novel NRPSs were identified in I89 strain, suggesting that A89 and B89 peptides do not have a nonribosomal origin.

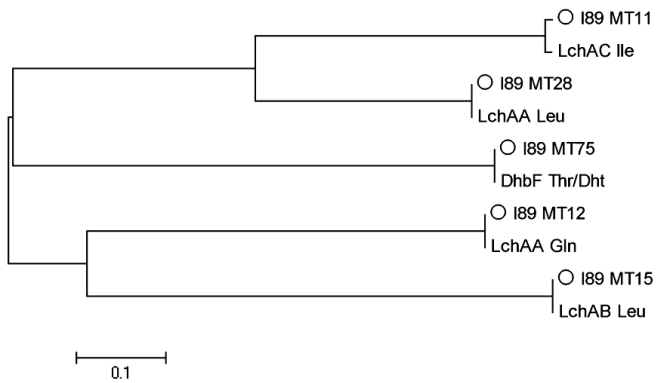


Figure 24: Phylogenetic analysis of the adenylation domains amplified from *B. licheniformis* I89. LchAA. LchAB and LchAC represent the three subunits of lichenysin peptide synthetase found in *B. licheniformis* ATCC 14580/DSM13 and DhbF the putative siderophore synthetase. The analysis was performed with the amino acid sequence between the core motifs A3 and A6 of each adenylation domain.

2.3 Discussion

The *Bacillus subtilis-licheniformis* group includes many strains used to manufacture industrial enzymes, antibiotics and biochemicals (Rey *et al.*, 2004). *B. licheniformis* strains have been widely described as a natural source of antimicrobial compounds produced both aerobically and anaerobically. Some examples include the nonribosomal antibiotic bacitracin (Johnson *et al.*, 1945) and the bacteriocin-like peptides like lichenin (Pattnaik *et al.*, 2001). Therefore, it was not surprising that *B. licheniformis* I89 was able to inhibit the growth of the Gram-positive *M. luteus*. Such ability promoted further investigation involving the antibacterials produced by this strain. A previous study described that I89 strain was able to produce and export to its supernatant the A89 antibacterial peptide when grown at 50 °C (Mendo *et al.*, 2004). Herein, it was also observed that *B. licheniformis* I89 colonies were able to inhibit the growth of *M. luteus* when both bacteria were incubated together at 37 °C. Moreover, it was also demonstrated by LC-ESI-MS analysis that the A89 peptide is also produced at this temperature. Therefore, the comparison of *B. licheniformis* I89 growth, sporulation and antagonistic activity when incubated at 37 °C and 50 °C was performed. At both of the tested conditions, antagonistic activity was first detected at the end of the exponential phase and the maximum activity was obtained approximately four hours later. The same profile was observed for *B. licheniformis* P40 and 490/5 strains, producers of bacillocin P40 and bacillocin 490 antibacterial peptides, respectively (Martirani *et al.*, 2002; Cladera-Olivera *et al.*, 2004b). The abolishment of activity after 36 hours of incubation of samples withdrawn from fermentations performed at 50 °C was not observed when I89 was incubated at 37 °C. Yet, a decrease of the antibacterial activity along the stationary phase of cultures incubated at 37 °C was visible. The reasons behind these findings could not be established so far. However it can be suggested that it might be due to the degradation of some of the antibacterial compounds by enzymes also produced by the I89 strain. It is possible that at 50 °C better conditions are met for the production and/or catalytic activity of such enzymes. Alternatively, the mixture of antibacterial compounds produced at 37 °C could be different from those produced at 50 °C, and consequently the stability to the prospective enzymatic activity could be distinct. Furthermore, it should be emphasized that fractions collected from I89 fermentations that occurred at 37 °C resulted in larger inhibition zones than those derived from fermentations at 50 °C. Thus, it appears that incubation at 37 °C is better for the production of I89 antibacterial compounds.

In the present study, production of spores was observed either at 37 °C or 50 °C. It is known that although not always directly dependent on each other, the production of secondary metabolites (e.g. antibiotics and proteases) and spore formation are often

synchronized (Hanlon and Hodges, 1981). Actually, in the present study the first spores were identified at the late exponential phase of growth, coinciding also with the beginning of antibacterial activity.

The supernatants of *B. licheniformis* I89 were found to be active against the Gram-positive strains of *L. monocytogenes*, *E. faecium*, *E. faecalis*, *H. influenzae* and *S. aureus*, including a MRSA clinical isolate, suggesting that I89 compounds exerts their activity in distinct targets. However, they were completely inactive against the Gram-negative bacteria tested so far. In fact, the majority of *B. licheniformis* antimicrobial compounds have Gram-positive bacteria or fungi generally associated with plants as preferential targets. Even so, bacillocin P40 is active against some Gram-negative microorganisms such as *Aeromonas* sp., *Enterobacter aerogenes* and *Pasteurella haemolytica*, which have been described as infection agents of economically important veterinary species, including fish (Cladera-Olivera *et al.*, 2004b).

It was not established before if the biological activity of *B. licheniformis* I89 supernatants was exclusively due to the production of the A89 peptide. Hence, in the present study the components of I89 supernatants were separated through the application of analytical procedures. It was found that *B. licheniformis* I89 produces other antibacterial compounds in addition to A89 peptide. Apart from A89 fraction, two other main fractions containing antibacterial compounds were identified. One of these fractions was not further characterized in the present study, whereas the other fraction consisting mainly of the B89 peptide (M= 3020 Da) and a compound(s) possessing M= 3038 Da were selected for additional studies. The molecular mass of the last compound(s) suggests that it could consist of a B89 variant possessing an additional water molecule. The pure A89 and B89 peptides were sequenced by Edman degradation. However, no information of their amino acidic content was obtained due to the earlier termination of the sequencing cycles. Edman degradation blockage has often been associated with the presence of dehydrated residues (Holtsmark *et al.*, 2006). Thus, it could be possible that A89 and B89 peptides contain such amino acids.

The LC-ESI-MS analysis of supernatants from *B. licheniformis* I89 cultures grown at 50 ºC revealed that B89 and its derivative (M= 3038 Da) were also produced at this temperature. Using this approach, it was not possible to identify the production of the well-known antibacterials bacitracin and lichenysin produced by certain *B. licheniformis* strains. Such results are in agreement with the fact that we were not able to identify A domains sharing high homology with the bacitracin operon within the *B. licheniformis* I89 genome. On the contrary, four from a total of seven A domains possessing high homology with the lichenysin peptide synthetase were identified. Lichenysins compounds differ in amino acid

composition at the positions Glx1, Asx5 and Ile/Leu/Val7 (Konz *et al.*, 1999). The adenylation domain of the I89 strain sharing high homology with the first adenylation domain of the lichenysin synthetase should be responsible for the activation of a Gln residue instead of a Glu, according to the nonribosomal code proposed by Stachelhaus *et al.* (1999). Therefore, the most probable scenario is that I89 strain would produce lichenysin D, as the *B. licheniformis* ATCC 10716 strain (Konz *et al.*, 1999). However, the medium composition, especially the carbon and nitrogen source are factors which significantly influence the production of those lipopeptides (Makkar and Cameotra, 2002). Therefore, unless the lichenysin NRPS present in I89 strain is non-functional, it might be able to produce this biosurfactant after optimization of the growth conditions. Like *B. licheniformis* ATCC 14580, I89 strain should contain a siderophore biosynthetic gene cluster, since MT75 A domain possessed homology with DhbF protein. The production and secretion of such molecules is part of a survival mechanism, which allows microorganisms to acquire iron when exposed to iron-limiting conditions. For instance, the siderophore SVK21 was isolated from *B. licheniformis* VK21 strain under iron deficiency conditions. According with the amino acid analysis and NMR spectrometry results, such compound was proved to be 2,3-dihydroxybenzoyl-glycyl-threonine (Temirov *et al.*, 2003). The analysis of MT75 adenylation domain indicates the activation of a Thr or a Dht residue. Therefore, it could be possible that under iron deficiency conditions the I89 strain can also produce the SVK21-like siderophore or a closely related compound.

A domains possibly incorporating novel NRPSs were not identified in the I89 strain. Therefore A89 and B89 peptides should not belong to the nonribosomally-synthesized group of peptides. In fact, it was recently demonstrated that these two peptides constitute the two-component lantibiotic lichenicidin (Begley *et al.*, 2009; Dischinger *et al.*, 2009; Shenkarev *et al.*, 2010). Lichenicidin is composed by the two peptides Bliα/Lchα and Bliβ/Lchβ, which have the same molecular masses and share the biological characteristics with the A89 and B89 peptides, respectively. For this reason, the nomenclature used in their first description (Bliα and Bliβ) will be adopted in the following chapters. Regarding the other lichenicidin producers (*B. licheniformis* ATCC 14580 and DSM13), it was found that these two peptides were exclusively identified in their cell wall associated fractions of cultures grown at 37 °C in LB medium or synthetic medium, respectively (Begley *et al.*, 2009; Dischinger *et al.*, 2009). Nonetheless, these lantibiotics were successfully detected in both supernatant and cell wall washes extracts of I89 grown in very similar conditions, since medium M composition is similar to that of the LB medium. The lantibiotic biological origin of Bliα and Bliβ can justify the inability to obtain their amino acid constitution by Edman degradation.

In conclusion, in this chapter we demonstrate that *B. licheniformis* I89 produces a broad range of natural compounds which can have relevant economical and industrial interests. Among these is the two-component lantibiotic lichenicidin. Bliα and Bliβ structure, biosynthesis and self-protection will be the major focus of this thesis and will be studied and discussed in more detail in the next chapters.

2.4 Experimental procedures

2.4.1 Bacterial strains and cultivation media

B. licheniformis I89 was first isolated from a hot spring in Azores island (Mendo *et al.*, 2000). *Enterococcus faecalis*, *Haemophilus influenzae*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* strains were isolated from patients of the Hospital Infante D. Pedro, SA – Aveiro. All of these strains were maintained in tryptic soy agar plates (TSA; Merck) and propagated at 37 °C. *Microcuccus luteus* ATCC 9341 was the indicator strain used to test antibacterials productions. Fermentations of *B. licheniformis* I89 were performed in medium M containing 10 g/L of NaCl, 10 g/L of trytptone, 5 g/L of yeast extract, 10 g/L of KH_2PO_4 and adjusted to a final pH of 6.5, adjusted with NaOH (Mendo *et al.*, 2004). When extraction of DNA was necessary, I89 strain was grown in tryptic soy broth (TSA, Merck).

2.4.2 Cell growth monitoring and antibacterials production

The monitoring of cell growth was performed in medium M at 37 °C and 50 °C. A pre-culture with approximately 16 hours of growth was always prepared. 1/100 volume of this pre-culture was used to inoculate fresh medium followed by incubation for 44 hours at 160 rpm. Every 4 hours, 3 mL were taken from the main culture and used to measure the following parameters: (a) bacterial density at OD_{600nm} (1 mL); (b) number of viable cells (500 μL); (c) supernatant and cell washes antibacterial activity (1 mL); (d) pH of the supernatant (e) production of spores.

2.4.2.1 Counting of viable cells

The total number of cells and spores present at each hour were evaluated using successive dilutions of the remaining culture in 1X PBS buffer (final volume of 1 mL). 50 μL of each dilution was platted on medium M agar plates and incubated overnight either at 37 °C or 50 °C. The total number of colonies present in each dilution was counted and the

respective values expressed in terms of CFU/mL, considering the volume and dilution that were plated.

2.4.2.2 Evaluation of antagonistic activity and pH determination

For each sampling point, 1 mL of culture was centrifuged at 12000 x g, for 5 min to separate the cells from the supernatant. A second centrifugation in the same conditions was performed to ensure that no cells were present. The pH of the supernatants was measured before their storage at -20 ºC. The pelleted cells were dissolved in 100 μL of a 70% solution of isopropanol/water, incubated at 4 ºC for 3 hours and finally centrifuged at 12000 x g for 5 min. The supernatant corresponding to the cell-wall wash fraction was transferred to a clean 1.5 mL microcentrifuge and stored at -20ºC. The antagonistic activity of both supernatant and cell wall washes fractions were tested by applying 30 μL of each sample to the bioassay plates' wells as described in section 2.4.8.

2.4.2.3 Production of spores

Since *B. licheniformis* I89 sporulates, the number of spores present at each hour of growth was calculated. This involved the incubation of 100 μL from the remaining 1 mL of culture for 20 min at 80 ºC. After this, successive dilutions were performed in 1X PBS buffer and 50 μL of each were platted in the agar plates of the corresponding medium and incubated overnight at 37 ºC or 50 ºC. The total number of colonies present in each dilution was counted and the respective values expressed in terms of CFU/mL, considering the volume and dilution that were plated. Each colony was considered to originate from the germination of one spore.

2.4.3 Extraction of genomic DNA from *B. licheniformis* I89

The extraction of genomic DNA from *B. licheniformis* I89 was performed with the Genomic DNA purification kit (Fermentas) from an overnight culture grown at 37 ºC. 4 mL of this culture were centrifuged for 5 min at 12000 x g, the pellet was resuspended in 200 μL of TE buffer, containing 10 mg/mL of lysozyme (Roche) and incubated for 30 min at 37 ºC. 400 μL of the Lysis Solution were added to the mixture, followed by incubation at 65 ºC for 5 min. At this time, 600 μL of chloroform were added and the solution gently emulsified by inversion. The sample was centrifuged for 2 min at 12000 x g and the upper aqueous phase transferred to a clean and sterile microtube. 800 μL of freshly prepared 1X Precipitation Solution were added, followed by several inversions of the tube approximately for 2 min. The mixture was centrifuged at 12000 x g for 2 min and the

supernatant completely removed. The pellet constituting the genomic DNA was gently dissolved in 100 μL of the provided NaCl solution (1.2 M), until the pellet was completely dissolved. 300 μL of cold ethanol were added and the genomic DNA was allowed to precipitate 10 min at -20 ºC. After centrifugation for 4 min at 12000 x g, the ethanol was removed and the pellet washed with 70 % ethanol. This solution was removed by centrifugation 5 min at 12000 x g and the pellet was allowed to dry completely in the flow chamber. Finally, the extracted DNA was dissolved in 100 μL of sterile distilled water.

2.4.4 Analysis of A domains in the *B. licheniformis* I89 genome

2.4.4.1 Construction of A domains library

Adenylation (A) domains were amplified from *B. licheniformis* I89 genomic DNA using the primers MTF (5'-GCNGGYGGYGCNTAYGTNCC-3') and MTR (5'-CCNCGDATYTTNACYTG-3') (Neilan *et al.*, 1999). The degenerate primers were constructed to target the A2 and A8 core motifs of the A domains, respectively. Each reaction was performed in a final volume of 25 μL using the Thermo Extensor High-Fidelity PCR master mix, with each primer final concentration of 1.5 pmol/μL. The amplification parameters were as follows: 94 ºC for 5 min, 35 cycles of 94 ºC for 30 sec, 51 ºC for 30 sec and 68 ºC for 1 min and a final extension step of 10 min at 68 ºC. Five independent PCR reactions were performed, mixed together, purified and eluted in a final elution volume of 30 μl with the GeneJET™ PCR Purification Kit (Fermentas), according to the manufacturer's instructions (Appendix 8). The purified PCR product was ligated to the pCR 2.1 plasmid (Appendix 3) using the TA cloning kit (Invitrogen). Briefly, the ligation reaction contained 7 μL of PCR product (approximately at 50-100 ng/μL), 25 ng of the pCR 2.1 plasmid, 4 U of ligase and 1X of ligase buffer in a final volume of 10 μL and incubated overnight at 14 ºC. 5 μL of this reaction were used to transform chemically competent *E. coli* DH5α cells, prepared as described in Appendix 4. Positive clones were selected based on kanamycin resistance and white/blue selection by incubation of LB agar containing 50 μg/mL of Kan and 40 μg/mL of X-Gal.

2.4.4.2 Sequencing of the A domains

Approximately 50 white colonies were randomly picked and the inserted fragment was amplified using the universal primers M13fw (5'- GTTTTCCCAGTCACGAC-3') and M13rv (5'-CAGGAAACAGCTATGAC-3'). Each reaction was performed in a final volume of 12.5 μl containing 1.5 mM $MgCl_2$, 200 μM dNTP's, 1X Green GoTaq® Buffer and 0.25 U of GoTaq®

Buffer (Promega), using a bacterial colony as DNA template. The amplification parameters were as follows: 94 ºC for 5 min, 35 cycles of 94 ºC for 30 sec, 50 ºC for 30 sec and 72 ºC for 1 min and a final extension step of 10 min at 72 ºC. The presence of the correct amplicons (approximately 1200 bp) was evaluated on 1 % agarose gel electrophoresis. Positive PCR products were subjected to restriction fragment length polymorphism (RFLP) in order to select distinct inserts before sequencing. Each RFLP reaction was prepared with 5 μL of each of these PCR reactions (without any purification step, approximately 0.1-0.5 μg of DNA), 1X buffer *TaqI* and 10 U of *TaqI* enzyme (Fermentas), in a final volume of 20 μL. After incubation at 65 ºC for 1 hour, each digestion was loaded on a 2 % agarose gel (in this step, the addition of loading buffer was not necessary). Electrophoresis was performed at 120 V during 1 hour.

The banding pattern was analyzed with the GelCompar II software (Applied Maths) and only one representative of each clustered group was submitted to nucleotide sequence determination (STABVIDA-Portugal).

2.4.4.3 Phylogenetic analysis of the I89 A domains

All the sequenced A domains were first analysed using BlastX program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The two sequences exhibiting the highest homology with the A domains obtained in this study were selected. Sequentially, all the deduced amino acid sequences between two conserved core motifs (A4 and A5) were aligned using the web-based software Clustal X. This alignment was the basis for the phylogenetic analysis performed with MEGA 4.1 software (Tamura *et al.*, 2007), using as parameters the Poison correction model for amino acids and a bootstrap consisting of 1000 replications as described by Stachelhaus *et al.* (1999).

2.4.5 Separation of antibacterial compounds from the supernatants of *B. licheniformis* I89 strain

B. licheniformis I89 strain was grown in medium M at 37 ºC with aeration (160 rpm) for approximately 16 hours. 1 mL of this culture was used to inoculate 1 L of fresh medium M and allowed to grow for another 16 hours in the same conditions. Subsequently, the cultures were centrifuged at 12000 x g for 10 min to remove the bacterial cells. The cell-free supernatant was transferred to a clean reservoir and 100 mL of 1-Butanol (Merck) was added. The mixture was stirred at room temperature for 1 hour. After this period the solution was centrifuged for 1 min at 4000 x g and the organic upper layer collected into a clean tube. The organic solvent was subsequently evaporated at 50 ºC for 16 hours in an

evaporator EZ2-Standard (GeneVac). The dried pellet was used for solid phase extraction procedure.

2.4.5.1 Solid phase extraction (SPE)

A 10 mg/mL solution of the dried pellet was prepared in 90% acetonitrile/H_2O and applied to a C8 solid-phase extraction (SPE) cartridge (Machenery-Nagel) previously conditioned with 100 % acetonitrile. The SPE cartridge matrix containing the sample attached was subsequently washed with the following solvent solutions: 100% dichloromethane, 100 % ethylacetate, 100 % acetone, 100 % acetonitrile, 90 % acetonitrile and finally 70 % acetonitrile. A vacuum of −0.5 bar was always applied during the procedure and all the solvents getting out of the cartridge were collected to distinct falcon tubes. The six different solvent solutions were evaporated at 50 °C for 16 hours in an evaporator EZ2-Standard (GeneVac). All the pellets were dissolved in 50 % acetonitrile and 50 μL were used for bioassay, as described in section 2.4.8. Following this procedure, it was found that antibacterial activity was exclusively detected in the 90 % and 70 % acetonitrile fractions. Therefore, in the following SPE procedures these two fractions were pooled together and concentrated by solvent evaporation as already described. The pelleted extract was further used for the preparative high-pressure liquid chromatography procedure described as follows.

2.4.5.2 prepHPLC of SPE extracts

100 mg of the SPE extract was resuspended in 1 mL of 50% acetonitrile solution and submitted to preparative high pressure liquid chromatography with the serie 1100 equipment (Agilent Technologies, Waldbronn, Germany) and a C18 column. The components separation was performed at a flow rate of 25 ml/min with detection at A= 210 nm using the following gradient parameters: 0–2 min (0 % solvent B), 2–3 min (0–40 % solvent B), 3–15 min (40–49 % solvent B) and 15 min–15 min and 10 seg (49–100 % solvent B), solvent A being ultrapure water containing 0.1 % formic acid and solvent B 100 % acetonitrile containing 0.1 % formic acid. All the fractions were automatically collected along the 20 min of the procedure, concentrated by complete solvent evaporation and finally resuspended in 1 mL of 50 % acetonitrile. 50 μL of each fraction were subjected to bioassay as described in section 2.4.8. According with the antibacterial activity and LC-ESI-MS results, three different fractions were defined. The fraction I was not analyzed. The fraction II corresponded to the pooling of vials containing compounds with retention times between 8.5 min and 11.3 min. The fraction III corresponded to the pooling of vials containing compounds with retention times between 12 min and 14.2 min. Fraction II and

III were concentrated by complete solvent evaporation and each of them was applied to a second prepHPLC procedure as described below.

2.4.5.3 prepHPLC of fraction II and III

Fraction II and fraction III obtained in the previous section were separately dissolved in 50 % acetonitrile to obtain a final concentration of 6 mg/mL. 1 mL of this solution was separated in the same preparative HPLC equipment as described in the previous section. However, the separation was performed at a flow rate of 25 mL/min with the following gradient: 2 min – 0 % solvent B, 3 min – 40 % solvent B, 35 min – 49 % solvent B, 35.5 min– 100 % solvent B and 40 min – 100 % solvent B. Solvent A consisted of ultra pure water containing 0.1 % of formic acid and solvent B being 100 % acetonitrile containing 0.1 % formic acid. All the 5 mL fractions obtained for the separation of fraction II and fraction III were concentrated by full evaporation of the solvent and resuspended in 1 mL of 50 % acetonitrile. 50 μL of each of the fractions were bioassayed as described in section 2.4.8. According to the antibacterial activity and LC-ESI-MS results, highly pure A89 and B89 peptides were obtained.

2.4.6 Mass spectrometry analysis

The analysis of the fractions was performed firstly by LC-ESI-MS on a qTof 2 hybrid quadrupol time of flight ESI mass spectrometer (Micromass/Waters, Milford, USA) coupled to an Agilent 1100 HPLC system (Agilent, Waldbronn, Germany). The volume injected for each analysis varied between 10 and 20 μL. Separation was performed with a Grom™ Sil 120 ODS-5 column from Grace with the following gradient: 5 min - 5 % solvent B, 20 min – 100 % solvent B, 25 min – 100 % solvent B, 26 min – 5 % solvent B and 30 min – 5 % solvent B at a flow rate of 0.2 mL/min. Solvent A being ultra pure water containing 0.1 % of formic acid and solvent B being 100 % acetonitrile containing 0.1 % formic acid. The qTof parameters were as follows: capillary at 3.0 KV, cone at 37 V, MCP of 1650 V, source temperature of 120 °C and dessolvation temperature of 320 °C.

2.4.7 Peptide sequencing

The purified A89/Bliα and B89/Bliβ peptides in the form of a dehydrated powder were subjected to sequence by automated Edman degradation at the Proteome Factory company (Berlin, Germany).

2.4.8 Antibacterial activity assays

Antibacterial activity was always assessed on TSA agar plates seeded with the indicator strain *M. luteus* ATCC 9341 at a final concentration of 0.02 (optical density at A= 600 nm). For colony bioassays, testing strains were directly streaked on the surface of the bioassay plates. For the analysis of liquid samples, wells (5 mm diameter) were made in the inoculated agar plates and filled with the appropriated volume for the assay. The plates were always incubated at 37 ºC overnight before interpretation and measuring of inhibition areas.

CHAPTER III

Morphological differentiation of *Bacillus licheniformis* I89

This chapter was partially submitted to publication at the FEMS Microbiology Letters journal:

"Characterization of two different morphological phenotypes of the lichenicidin producer *B. licheniformis* I89"

Tânia Caetano, Andreia Gomes, Roderich D. Süssmuth, Sónia Mendo

3.1 Introduction

The Koch's postulates opened the era of single-cell, pure-culture microbiology. Once isolated, a certain bacterial strain is grown and its population reaches a certain density by the uptake of available nutrients (Lopez *et al.*, 2009). All of the cells present in such a population possess identical genomes and their subculturing should perpetuate its clonal ancestor characteristics. However, along this process, bacterial dissociation has been observed. This phenomenon consists in the emergence of variants of the same bacterial strain, differing in a number of characteristics at the morphological, physiological and/or genetic level (Golovlev, 1998; Tsygankova *et al.*, 2004).

B. licheniformis I89 is an antibacterial producer, including the recently characterized lantibiotic lichenicidin (Begley *et al.*, 2009; Bierbaum and Sahl, 2009; Shenkarev *et al.*, 2010). In the course of *B. licheniformis* I89 culturing process, two distinct morphological colony types were detected. For *B. licheniformis*, the phenotype transition has been described as a change of its growth phase (Bisset and Street, 1973). In other cases it is induced. For instance, the subculturing on nutrient broth of a 7-day-old *B. licheniformis* 103 strain, previously grown under sporulation suppression media revealed its dissociation into several distinct phenotype variants (Tsygankova *et al.*, 2004).

The phenotype metastability has been described in several Gram-positive and Gram-negative specimens, including members of *Bacillus*, *Staphylococcus*, *Escherichia* and *Actinobacillus* genus (Fine *et al.*, 1999; Kravchenko *et al.*, 2000; Doroshenko *et al.*, 2001; Il'inskaya *et al.*, 2002). Nevertheless, the reasons triggering such adaptative changes remain unknown, despite the efforts drove over the last three decades. For instance, the subculturing of the rough-type *Actinobacillus actinomycetemcomitans* in a daily basis promoted its conversion to a turbid smooth phenotype. Several studies showed the differences in the fimbriae abundance, *in vitro* ability of epithelial cells invasion and also

the expression of outer membrane proteins between the two morphological variants (Inouye *et al.*, 1990; Meyer *et al.*, 1991; Haase *et al.*, 1999). For *B. stearothermophilus* NCA1518 morphological variants, it was found that the same nutritional and biochemical capabilities were kept, despite the suspicion of variation in the heat resistance of their spores (Humbert *et al.*, 1972).

The phenotype instability of bacterial strains can have a negative impact in biotechnological applications, since industrially useful properties can be lost in the process (Berditsch *et al.*, 2007). A good example of that relies on the gramicidin S (Gause and Brazhnikova, 1944) antibiotic producer *Aneurinibacillus migulans* ATCC 9999 (*Bacillus brevis* var. G.B.). A rough appearance characterized the original colonies of this strain. However, during the early applications for gramicidin S industrial production, several distinct phenotypes variants, which originated spontaneously from this strain, were discovered. Intriguingly, two of such colonies lost their ability to produce gramicidin S (Zharikova *et al.*, 1964; Berditsch *et al.*, 2007). On the other hand, some interesting and useful properties can also arise from this variability. One of such examples is the attenuated *Bacillus* Calmette-Guérin (BCG) ancestral strain, which was obtained by Albert Calmette and Camille Guérin through repeated subculturing of *Mycobacterium bovis*, responsible for bovine tuberculosis, on a glycerin-bile-potato mixture (Calmette, 1927). The divergent methods of striking and storage of the BCG strain along the years also originated many substrains showing phenotypic and genetic heterogeneity (Bedwell *et al.*, 2001).Such diversification was accompanied by the deletion of some genetic regions (RD) which in some cases affected the antigenic content (Castillo-Rodal *et al.*, 2006).

Here, we confirmed through molecular-based techniques that both morphological variants detected in this study were spontaneously derived from *B. licheniformis* I89 ancestor strain. The growth and sporulation activity of each variant were investigated on two different cultivation media often used for *B. licheniformis* I89 culturing in our laboratory. Since phenotype instability was associated to the non-production of active compounds in the past, the production of lichenicidin peptides (Bliα and Bliβ) by each I89 variant was also investigated by LC-ESI-MS analysis.

3.2 Results

3.2.1 Changes in *B. licheniformis* I89 colony morphology

In the course of the present study, *B. licheniformis* I89 was routinely subcultured and maintained at 37 ºC on tryptic soy agar (TSA) plates. The *B. licheniformis* I89 phenotype was characterized by protrusive circular colonies with a central rough area surrounded by

defined borders. These cells strongly adhered to the agar, appearing partially embedded in it (Figure 25A). During a routine procedure, 2-days-old liquid cultures were inoculated on TSA plates and incubated overnight at 37 ºC to monitor the existence of potential contaminations. After this incubation period, colonies with two different morphologies could be observed: the original rough (R) colonies, strongly embedded in the agar and a novel colony type, consisting of non-agar embedded colonies with a smooth (S) appearance that consisted on a central surface delimited by swarming-like borders (Figure 25B). The inner section of these S-type cells could be easily removed from the agar, while the limiting border cells were difficult to detach. Moreover, the R-type variant presented a whitish-dry aspect when compared with the S-type variant. Multiple colony morphology is a phenomenon commonly described for some microorganisms such as *A. migulanus* and *Clostridium acetobutylicum* (Adler and Crow, 1987; Berditsch *et al.*, 2007). Even so, the S-type phenotype was at first believed to be a contaminant-borne microorganism. To elucidate this, different molecular procedures were applied to both variants and respective antibacterial activity tested.

The amplification and sequencing of the 16S rDNA gene of both variants showed that their sequences shared 100 % homology at the nucleotide level. Their comparison with the nucleotide database through the BLAST web-based software confirmed their classification as *B. licheniformis* specie.

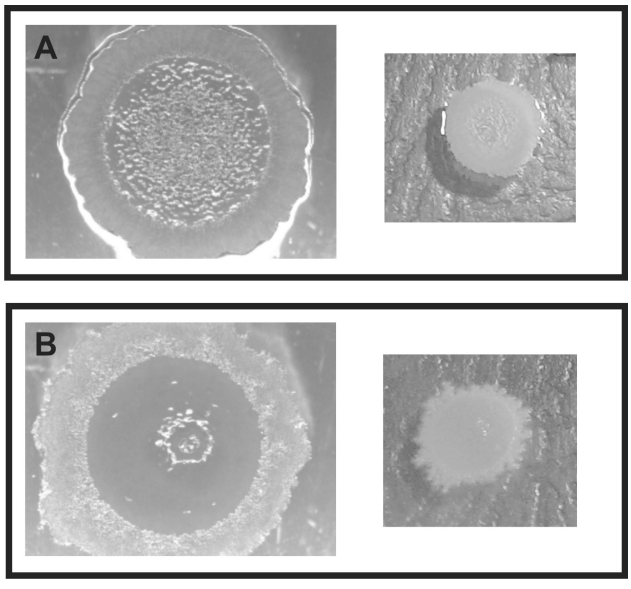


Figure 25: Different colony morphologies identified in *B. licheniformis* I89. The distinctive rough (A) and smooth (B) phenotypes were isolated from this strain.

Simultaneously, the genetic relationship between the variants was investigated by PCR-based methods of fingerprinting. Such methodologies take advantage of the repetitive sequences spread through the bacterial genome and the obtained fingerprints resemble "bar code" patterns analogous to UPC codes used in grocery stores (Lupski, 1993; Rademaker and DeBruijn, 1997). Here, rep-PCR analysis was performed by the application of the BOXA1R and ERIC primers (Rademaker and DeBruijn, 1997). This analysis showed that both R- and S-type colonies exhibited the same band profile (Figure 26). However, the pattern retrieved by *B. licheniformis* ATCC 10716 strain was distinctively different, even though some common bands could be observed by the BOX-PCR analysis. Thus, the 16S rDNA and rep-PCR results confirmed that R- and S-type colonies should constitute *B. licheniformis* I89 variants. Occasionally, rep-PCR analysis allows the recognition of deletions and/or insertions on the bacterial chromosome. However, such rearrangements at the DNA level were not detected by either of the methods employed.

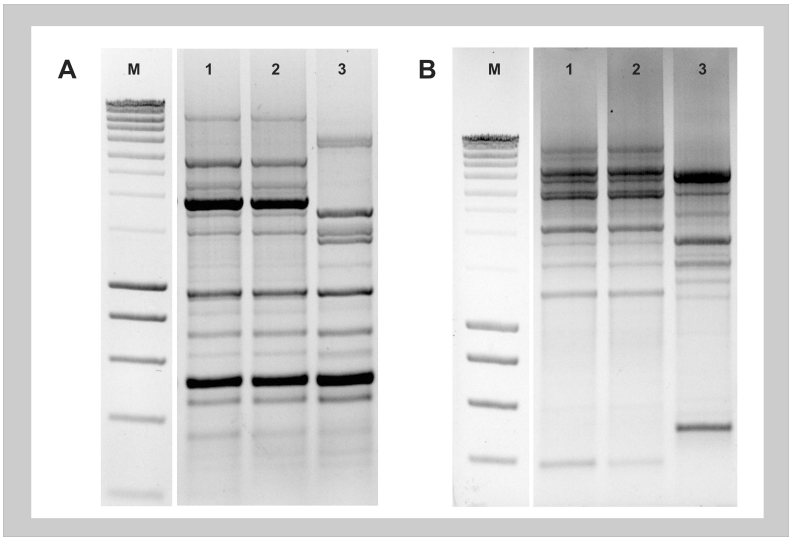


Figure 26: BOX-PCR (A) and ERIC-PCR (B) profiles obtained for *B. licheniformis* I89 rough (1) and smooth (2) phenotypes. The *B. licheniformis* ATCC 10716 strain was also included in the analysis (3). M represents the DNA molecular marker.

The capability to affect *M. luteus* growth of each variant was first evaluated through colony-based-bioassays. It was found that both R- and S-type colonies maintained their antibacterial activity against *M. luteus* (Figure 27). Interestingly, the activity observed around the S-type variant seemed slightly weaker when compared with that of the R-type variant (Figure 27B). The phenotype reversion possibility was tested by successively subculturing each variant on TSA plates incubated at 37 ºC. It was found that under this

circunstances, morphological properties of S-type did not revert to the parental R-type phenotype.

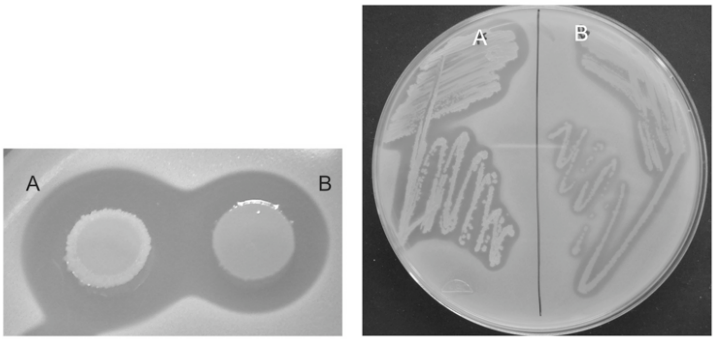


Figure 27: *B. licheniformis* I89 rough (A) and smooth (B) variants showing antibacterial activity against the *M luteus* indicator strain.

3.2.2 Growth and antagonistic activity of *B. licheniformis* I89 different phenotypes

The phenotypic variation is often associated with microbial adaptation to the laboratory environment. The bacterial fitness, growth profile and sporulation activity are occasionally affected in such processes. Thus, the identification of hypothetical differences on the growth rate, sporulation and antagonistic activity of both R- and S-type colonies was investigated over 44 hours (Figure 28). All the assays were performed at 37 ºC, since this was the temperature where phenotypic dissociation was first observed. Results showed that no differences on biomass production between R- and S-type colonies could be detected over time of growth in both media tested (Figure 28A). Also, under the tested conditions the growth kinetics of two-phenotype variants was characterized by a lag-phase of approximately 4 hours, followed by an exponential phase of about 16 hours. The stationary phase was reached approximately after 20 and 24 hours of growth on TSB and medium M, respectively. Similarly, sporulation experiments showed no major differences between R- and S-type variants, when the same culture medium was used (Figure 28B). When the cultures were performed in TSB medium, spores could be detected after 8 hours of growth in both variants. Under these conditions, spores were slowly formed until 20 hours of growth. After this period their biomass values increased exponentially until the 32 hours of incubation. In medium M, the spores were only detected after 16 hours of incubation in both R- and S-type cells. Under such conditions, the accumulation of the biomass increased exponentially until 32 hours of growth. These results suggested that the triggering of the sporulation process should be influenced by the nutritional contribution from the cultivation medium.

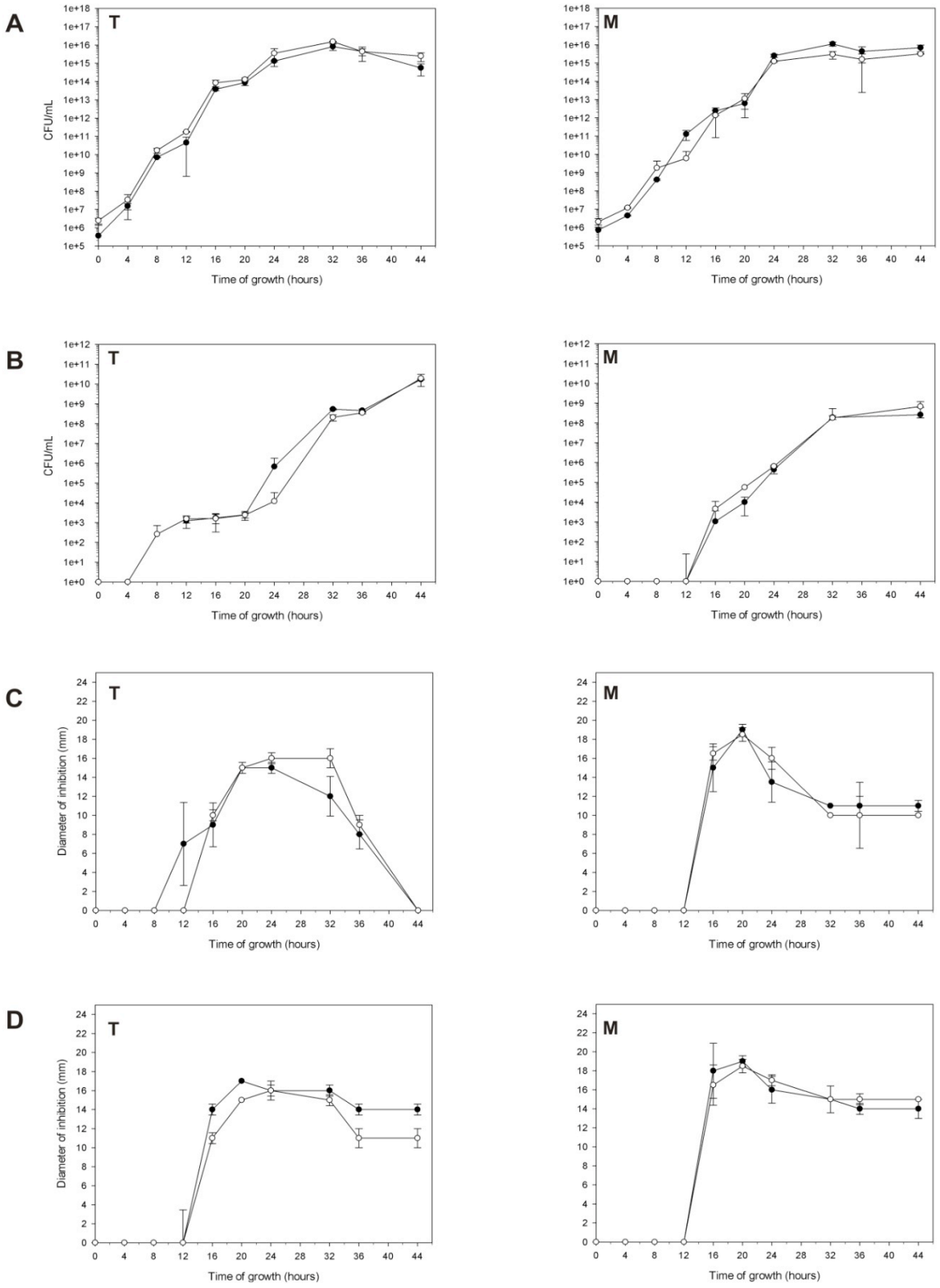


Figure 28: Bacterial growth (A), sporulation (B) and antibacterial activity associated with the supernatant (C) and the cell-wall washes fractions (D) of rough (black circles) and smooth (white circles) phenotypes in medium M (M) and tryptic soy broth (T).

Regarding the antibacterial production, it was observed that in TSB medium, *M. luteus* inhibition in the supernatant as well as cell-wall washes of the R- and S-type colonies was detected after 16 hours of growth (Figure 28C/D). In this medium, the maximal antibacterial activity was achieved between 20 and 24 hours of incubation in both fractions of each of the phenotypic variants. Moreover, after this period, the supernatant bioactivity clearly decreased until no activity was observed after 44 hours. A similar behavior was observed on the inhibition zone diameter from cell wall washes. However inactive fractions were not detected, even after 44 hours of growth. Alike to what was observed in TSB medium, the antagonistic activity of the analyzed fractions in medium M was first detected after 16 hours of growth on both R- and S- phenotypes. In this culture medium, the maximum activity of the supernatant and cell wall washes was observed at 20 hours of growth. After this stage, the bioactivity decreased but was never null, contrary to what was observed with TSB supernatant fractions.

3.2.3 Lichenicidin production by *B. licheniformis* I89 phenotypic variants

The production of lichenicidin peptides Bliα and Bliβ by the two morphological variants was evaluated by LC-ESI-MS analysis of total n-butanol extracts after 20 hours of growth in both TSB and medium M (Figure 29).

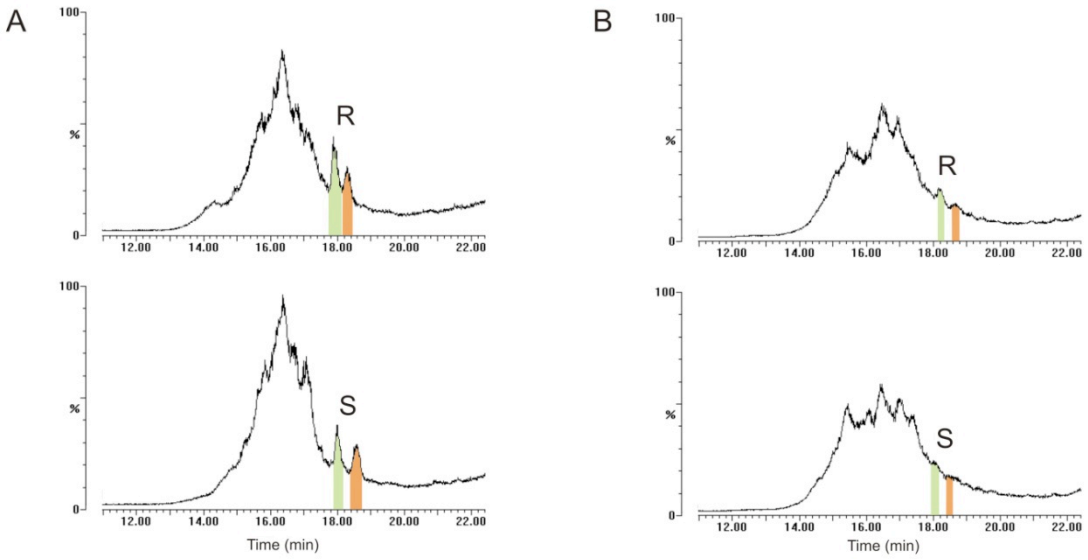


Figure 29: TIC chromatogram of total butanol extracts from rough (R) and smooth (S) variants grown in medium M (A) or TSB (B) after 20 hours of incubation. The Bliα and Bliβ peptides were identified in the green and orange area, respectively.

The total ion chromatogram obtained for medium M extracts showed two defined peaks between the R_t= 18 min and R_t= 19 min for both R- and S- variants. In the first peak the Bliα peptide (M= 3250 Da) was detected, while the second peak corresponded to the peptides Bliβ (M= 3020 Da) and Bliβ with an additional water molecule (M= 3038 Da; Bliβ$^+$) (Figure 29A). These peptides were also successfully identified on the supernatants of TSB cultures; the peaks corresponding to these peptides although in low abundance and less defined, had the same retention time as those obtained when samples produced on medium M were analyzed (Figure 29B). When cultured in medium M, no major differences were observed between the chromatogram profile of R- and S- phenotypes. On the contrary, the LC-ESI-MS analysis of TSB extracts revealed some discrepancies such as the abundance of lichenicidin peptides peaks, that were lower in the S variant extract.

The relative abundance of the lichenicidin peptides was quantified with the values obtained from TICs signals (total ion chromatograms) by integration of each peak, considering double and triple charged molecule peaks. In TSB medium (Figure 30B) the values obtained for the occurrence of Bliα peptide on S-type (137.2 ± 5.7 AU; AU=arbitrary units) extracts were three times lower than those of the R-type extracts (397.0 ± 45.8 AU). Likewise, the Bliβ and Bliβ$^+$ peptides were also less abundant in the S-type extracts (34.0 ± 10.2 AU and 27.8 ± 6.9 AU, respectively) than in the R- phenotype extracts (Bliβ: 54.7 ± 13.8 AU; Bliβ+: 50.0 ± 13.2 AU). In medium M (Figure 30A), the abundance of Bliα peptide did not differ greatly between the R- (944.2 ± 298.0 AU) and the S- variant (871.49 ± 118.8 AU) extracts. Regarding the other two peptides, higher abundance values were obtained from the R variant extracts (Bliβ: 374.3 ± 123.8 AU; Bliβ$^+$: 307.7 ± 77.9 AU) when compared to the sample extracts from S-type cultures (Bliβ: 288.3 ± 123.8 AU; Bliβ$^+$: 183.0 ± 21.1 AU).

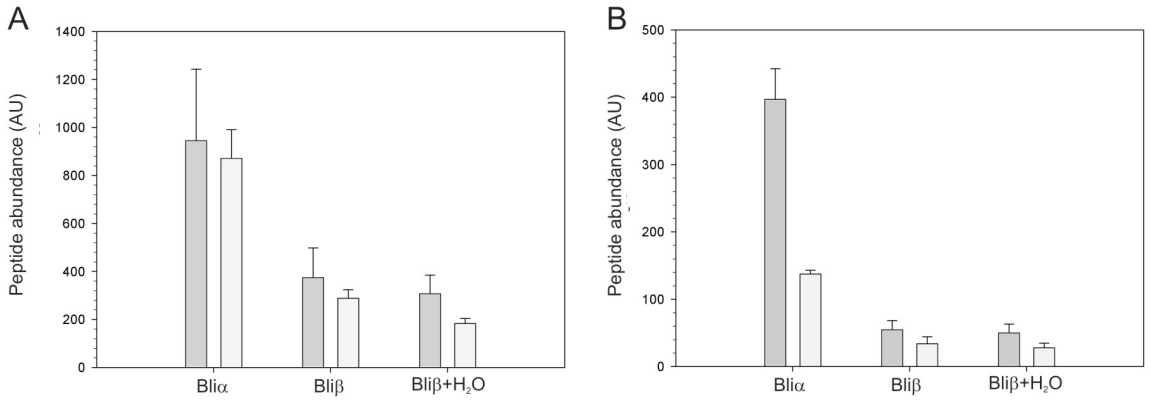


Figure 30: Comparison of relative abundance of lichenicidin peptides on butanol extracts of rough (dark grey) and smooth (light grey) variants in medium M (A) and TSB (B), after 20 hours of growth. Measurements were performed in triplicate.

3.2.4 Total protein analysis of rough and smooth phenotypes

The abundance of lichenicidin Bliα peptide was substantially lower in TSB cultures of the S-type *B. licheniformis* I89 variant. Such difference was not so evident when medium M was used as the cultivation medium. Therefore, it was decided to analyze total protein profiles of R- and S-type phenotypes grown in TSB. The cells from both cultures were harvested and suspended in 1X PBS buffer, sonicated, briefly centrifuged and finally examined by SDS-PAGE (Figure 31). The visual comparison of both profiles did not reveal major differences between proteins expressed by R- and S-type phenotypes. However, bands with higher intensity were detected around 46 kDa and 30 kDa region of the pattern banding obtained from S-type phenotype, when compared with the same region of R- variant. In opposition, some bands with lower molecular weight (between 17 and 25 kDa) and a little more than 30 kDa seemed to be more intense on the total protein analysis of the R- variant.

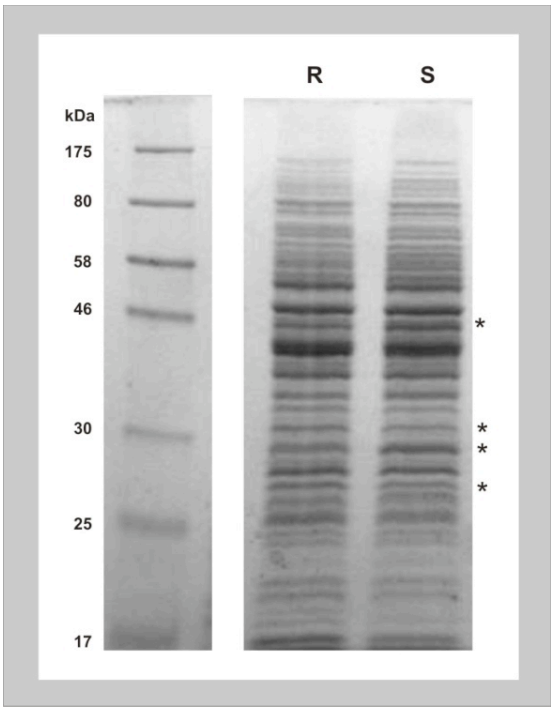


Figure 31: SDS-PAGE gel of total proteins from *B. licheniformis* I89 rough (R) and smooth (S) variants grown in TSB medium.

3.3 Discussion

The bacterial dissociation term was applied by de Kruif (1921) to denote the appearance of a variable colonial population derived from a pure bacterial culture. Generally, such variants are sufficiently stable to maintain their characteristics over several

generations. However, some other types can sporadically reverse to the appearance of the parental strain (Braun, 1947). Dissociation phenomenon was recognized for *B. licheniformis* I89 when 2-days-old cultures were subcultured on TSA plates and incubated at 37 ºC. In these plates, R- colonies could be observed showing the same morphology of the parental I89 strain, but colonies with a new S-type phenotype could also be observed. Previously, it has been described that small variations in medium composition, incubation temperature and agar content of the solid medium can influence the colony morphology of *B. subtilis* (Veening *et al.*, 2006). However, since *B. licheniformis* I89 R- and S-type colonies were first observed in the same plate, these changes could not be attributed to those factors. 16S rDNA gene amplification and sequencing allowed the classification of both R and S-type phenotypes as members of the *B. licheniformis* specie. Furthermore, their phylogenetic relationship was investigated by rep-PCR. This technique proved to be useful in the subtyping of some *Bacillus* species, including *B. licheniformis* (de Clerck and de Vos, 2004; Whitaker *et al.*, 2005). The exact same fingerprint profile was obtained for R- and S-type colonies, and yet perfectly distinguishable from that of *B. licheniformis* ATCC 10716. The evidence provided by these results excluded the possibility of bacterial contamination, suggesting that both morphological phenotypes arose spontaneously from the ancestral *B. licheniformis* I89 strain. Moreover, the reversion to the initial R- phenotype was not observed after subsequent passages of S- colonies, suggesting the stability of the dissociation. Such maintenance was also observed for five different phenotype variants of *B. licheniformis* 103 (Tsygankova *et al.*, 2004). Nevertheless, we believe that reversion of S- to R-type colonies can still occur as a response to external conditions such as a different incubation temperature or a different media composition. For instance, the laboratory strain *B. subtilis* 168 is still capable of forming colonies with vein-like structures formed by elevated bundles of cells when cultivated on minimal medium agar plates (Veening *et al.*, 2006).

B. licheniformis I89 was first described and characterized due to its ability to produce antibacterial compounds able to inhibit the growth of *M. luteus*, including the lantibiotic lichenicidin. Therefore, the impact of the I89 strain phenotypic switch on growth, sporulation and antibacterial activity was evaluated at 37 ºC. Two different culture media were selected for the assays: i) medium M, traditionally used for *B. licheniformis* I89 antibiotic production (Mendo *et al.*, 2004) and ii) TSB, a general purpose medium, which is equivalent to the agar medium used for colony-bioassay and where I89 strain morphological differentiation was initially detected. Results showed that R- and S-type variants produced the same biomass during the growth in both culture media. Also, the

switching of R- to S-type morphology was not accompanied by loss of sporulation ability, as previously observed for other industrial important strains, as the acetone and butanol-producer *C. acetobutylicum* (Adler and Crow, 1987; Berditsch *et al.*, 2007). Nevertheless, sporulation activity seems to be initiated earlier when the strains were grown in TSB medium (Figure 28B). Regarding the antibacterial activity, no major differences were detected between R- and S-type phenotypes grown in medium M. On the other hand, cell wall washes bioactivity from R-type colonies grown in TSB were in general superior to those of S-type colonies. The same tendency was not observed for supernatant fractions. Among the bioactive compounds produced by I89 strain, our major interest relies on the production of the lantibiotic lichenicidin. LC-ESI-MS analysis of total n-butanol extracts revealed that Bliα, Bliβ and Bliβ$^+$ (Bliβ plus one additional water molecule) are produced by R and S phenotypes grown in both culture media. However, a quantitative analysis of peptide production by these variants showed that their abundance is generally lower in S-type extracts. This aspect was particularly evident with the Bliα peptide detected on the extracts of TSB cultures. Recently it was experimentally shown that Bliα and Bliβ peptides can interact synergistically to exert their antagonist activities (Shenkarev *et al.*, 2010). Thus, the production results could be related with the decreased bioactivity observed on the cell wall associated fractions from the S-type colonies when compared with those of the R-type colonies grown in TSB medium.

The decrease and even the abolishment of antibacterial production was previously associated with the morphological dissociation phenomenon. For instance, the gramicidin S producer parental strain *A. migulanus* exhibited a rough-like phenotype dissociated into six different phenotypes, two of them possessing a smooth morphology. Among these, the S-type colonies lost the ability to produce the nonribosomally-synthesized antibiotic gramicidin S (Berditsch *et al.*, 2007). Also, to the best of our knowledge this is the first description of bacterial dissociation phenomenon possibly related with an impact on lantibiotic production, namely the two-component lantibiotic lichenicidin.

The phenotypic differences have also been associated with the occurrence of deletions and/or insertions on the chromosome or also with molecular mechanisms as differential gene expression of one or more genes (Honda *et al.*, 2006; Berditsch *et al.*, 2007). Anyhow, in such complex biological processes differences in protein expression should always been expected. With this in mind, a preliminary analysis of protein expression by the R- and S-type colonies in TSB medium was performed. Despite the complexity of the protein banding profile obtained, some differences could be visually identified. This study constituted a first approach for future investigations, which should

involve the optimization of the protein separation methodologies (possibly the application of 2D-gel electrophoresis), followed by protein identification in order to understand the key processes behind colony differentiation of the *B. licheniformis* I89 parental strain.

Overall, the results obtained suggest that *B. licheniformis* I89 dissociation implied the transition of a rough and more complex to an apparently simple and smooth colony morphology on TSA plates. The growth of bacteria under laboratory conditions often culminates in the selection of strains in which the robustness of colony morphology is dramatically diminished (Aguilar *et al.*, 2007). This process is often referred as 'domestication' (Branda *et al.*, 2001; Aguilar *et al.*, 2007). Thus, S-type colonies could correspond to a stage of *B. licheniformis* I89 'domestication' process, which eventually can culminate on a lichenicidin-deficient strain, when permanently cultivated on media such as TSB and/or TSA. *B. licheniformis* is a biotechnologically important microorganism since numerous commercial and agricultural applications are known for its extracellular products (Rey *et al.*, 2004; Veith *et al.*, 2004). Therefore, the pursuit and assembly of pieces involved on its morphological dissociation could be a useful tool to avoid its potentially negative impacts.

3.4 Experimental procedures

3.4.1 Bacterial strains and cultivation media

Two different liquid culture media were routinely used for the cultivation of *B. licheniformis* I89: a) tryptic soy broth (TSB) (Merck); b) medium M (Mendo *et al.*, 2004), constituted by 10.0 g/L of Bacto tryptone, 5.0 g/L of yeast extract, 10.0 g/L of NaCl and 10.0 g/L of KH_2PO_4 and pH adjusted to 6.5 at 25 ºC (with 1M NaOH). For the cultivation on agar plates, 20 g/L of microbiological agar (Sigma) was added to the respective liquid culture medium before autoclaving. *B. licheniformis* ATCC 10716 and *M. luteus* ATCC 9341 were maintained on TSA plates.

3.4.2 Genetic analysis

Identification of *B. licheniformis* I89 subtypes was performed by amplification and sequencing of the 16S rRNA gene. For the amplification, the following primers were used: 27F (5'-AGAGTTT GATCMTGGCTCAG-3') and 1492R (5'-TACCTTGTTACGACTT-3') (Lane, 1991). Each reaction was performed in a final volume of 50 μL using the Thermo Extensor High-Fidelity PCR master mix, with each primer final concentration of 1.5 pmol/μL. The

amplification parameters were as follows: 94 ºC for 5 min, 35 cycles of 94 ºC for 30 sec, 56 ºC for 30 sec and 68 ºC for 2 min and a final extension step of 10 min at 68 ºC. Bacterial suspensions were performed in 20 μL of sterile distilled water and 1 μL of this solution was used as DNA template for the amplification. The resulting amplicons of approximately 1600 bp were submitted to sequencing reaction. The nucleotide sequences were compared to those available in the GenBank database using the BLAST web-based software (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

The genetic relationship of the two morphologically distinct colonies was investigated by repetitive extragenic palindromic-PCR (rep-PCR), using the primer BOXA1R (5'-CTA CGGCAAGGCGACGCTGACG-3') and the two ERIC primers ERIC1R (5'-ATGTAAGCTCCTGGGGATTCAC-3') and ERIC2 (5'-AAGTAAGTGACTGGGGTGAGCG-3') (Rademaker and DeBruijn, 1997). Each BOX or ERIC reaction was performed in a final volume of 25μl containing 1.5 mM $MgCl_2$, 200 μM dNTP's, 1X Green GoTaq® buffer and 0.25 U of GoTaq® Buffer (Promega) and 0.6 pmol/μL of BOXA1R or 0.3 pmol/μL of each ERIC primer. 1 μL of a bacterial suspension prepared as abovementioned was used as DNA template to the PCR reaction. The amplification parameters were as follows: 94 ºC for 5 min, 35 cycles of 94 ºC for 30 sec, 50 ºC for 30 sec and 72 ºC for 8 min and a final extension step of 10 min at 72 ºC. The amplicons were separated in a 1 % agarose gel in 1x Tris-acetate-EDTA buffer (Bio-Rad) at a constant voltage of 100 V.

3.4.3 Monitoring of bacterial growth and spore production

The monitoring of cell growth was performed in medium M and TSB at 37 ºC. A pre-culture with approximately 16 hours of growth was always prepared. 1/100 volume of this pre-culture was used to inoculate fresh medium followed by incubation for 44 hours at 160 rpm. Every 4 hours, aliquots were withdrawn from this culture and used to determine the total number of viable cells, to measure the bacterial density at OD_{600nm} and for the evaluation of spores' production. The total viable counts were obtained by successively diluting the culture in 1X PBS buffer (final volume of 1 mL) and plating each dilution in agar plates of the corresponding culture medium (either TSB or Medium M). After 18 hours of incubation at 37 ºC the total number of colonies was calculated and expressed in terms of CFU/mL. Spores production was estimated by incubation of 100 μL of the bacterial culture at 80 ºC for 20 min (Berditsch *et al.*, 2007). Successive dilutions of this suspension were performed in cold 1X PBS buffer and 50 μL of each dilution were platted on TSB or

medium M agar plates. After 18 hours of incubation at 37 ºC the colonies present in each plate were enumerated and assumed to originate from the germination of a single spore.

3.4.4 Antagonistic activity monitoring

From samples collected at different time intervals, the bacterial cells were separated from the supernatant by centrifugation of 1 mL of the culture at 13,500 x g for 5 min. The obtained supernatant was centrifuged again, in the same conditions, to remove any vestigial cells. The culture supernatant was transferred to a new microcentrifuge tube and stored at -20 ºC. The pelleted cells were dissolved in 100 μL of a 70 % solution of isopropanol, incubated at 4 ºC for 3 hours and centrifuged at 13,500 x g for 5 min. The obtained supernatant constituted the cell-wall wash, which was transferred to a clean microcentrifuge tube and stored at -20ºC. 30 μL of the supernatant and cell wall washes fraction were applied to bioassay plates to test their antagonistic activity.

3.4.5 Lichenicidin production evaluation

For lichenicidin production evaluation by LC-ESI-MS a pre-culture of each strain was performed in the appropriate medium and allowed to grow at 37 ºC, with aeration for approximately 16 hours. 500 μL of these cultures were used to inoculate 50 mL of fresh medium (either medium M or TSB) and grown at 37 ºC with aeration (160 rpm) for 20 hours. Total extracts were obtained by the addition of 5 mL of 1-Butanol (Merck) to 20 mL of each culture. The solution was sonicated for 15 min, stirred for 1 hour at room temperature and finally centrifuged at 5,000 x g for 5 min. The upper organic phase was collected and its solvent subsequently evaporated at 50 ºC for 16 hours in an evaporator EZ2-Standard (GeneVac). The pellet was dissolved in 1 mL of 50 % acetonitrile before LC-ESI-MS analysis.

3.4.6 Total protein analysis

B. licheniformis I89 strains were grown either in medium M or TSB as previously described (section 3.4.3) for 10 hours. 1 mL of culture was centrifuged for 5 min at 12,000 x g with refrigeration of 4 ºC. The supernatant was taken out and the pellet centrifuged again in the same conditions for 1 min to completely remove the remaining supernatant and the pellet was store at -80 ºC until further use. This procedure was performed in triplicate for each cultivation medium tested.

Each pellet was dissolved in 100 μL of ice cold 1X PBS and sonicated in a cold water bath 5 times for 10 sec with intervals of 10 sec. The protein sample was mixed with the 5X

sampling buffer in a proportion of 4:1 (v/v). The mixture was boiled in water for 10 min and 10 μL of each sample was loaded into the gel. The glycine-SDS-PAGE protocol herein described was adapted from Laemmli (1970). Briefly, the polyacrylamide gels used were prepared as described in Table 5. The running buffer (1X) was added to the anode and cathode electrodes and the samples were loaded. The electrophoresis was started with an initial voltage of 30 V until the samples entered completely into the stacking gel and changed to 100 V until the blue dye front reached the bottom of the gel. Proteins were visualized directly in the gels by Comassie staining. In this procedure, the gels were incubated in 100 mL of Fixing Solution for 15 min with gentle shake. Subsequently, the gels were washed with distilled water 5 times over a period of 1 hour, with gentle shaking between washes. Finally, 100 mL of the Staining Solution was added and the gels were incubated at room temperature with agitation until visible protein bands appeared (approximately 30 min). For better results the gels were left in distilled water overnight with gentle shaking before image acquisition.

Table 5: Reagents used for the preparation of monomer solutions of the gels used in this section. APS stands for ammonium persulphate and TEMED for N,N,N',N'-tetramethylethylenediamine. These two compounds should be added immediately prior to pouring the gel.

Solutions/Reagents		Stacking gel (4 %)	Separating gel (15 %)
AB-3	(mL)	0.67	7.5
1.5 M Tris-HCl pH 8.8	(mL)	-	3.75
0.5 M Tris-HCl pH 6.8	(mL)	1.25	-
20% SDS	(μL)	25	75
10% APS	(μL)	25	75
TEMED	(μL)	5	10
Distilled water	(mL)	3.07	3.6
Total volume	(mL)	5	15

Solutions

5X sampling buffer: 10 %SDS, 10mM β-mercapto-ethanol, 20 % glycerol, 0.2 M Tris-HCl [pH 6.8], 0.05% bromophenolblue)

AB-3 solution: Dissolve 48 g of acrylamide and 1.5 g of bisacrylamide in 100 mL of distilled water.

1X Running buffer: 25 mM Tris-HCl, 200 mM glycine, 0.1% (w/v) SDS, pH 8.0 .

Fixing Solution: 200 mL of 40% ethanol and 50 mL of 10% of acid acetic, in a final volume of 250 mL.

Staining Solution: 4 mL of stock dye solution, 8 mL of phosphoric acid and 3.75 mL of ethanol, in a final volume of 100 mL.

Stock dye solution: 0.33 g of Comassie Blue G powder diluted in 100 mL of a phosphoric acid:ethanol (2:1) solution. The solution should be stored in a brown bottle and stored in the dark.

3.4.7 Mass spectrometry analysis and antagonistic activity

The samples were subjected to LC-ESI-MS analysis as described in section 2.4.6 (page 60). When required the antibacterial activity assays were performed according to section 2.4.8 (page 61).

CHAPTER IV

Heterologous expression of lichenicidin in *Escherichia coli*

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"Heterologous expression, biosynthesis and mutagenesis of type II lantibiotics from *Bacillus licheniformis* in *Escherichia coli*"

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4.1 Introduction

Bacillus licheniformis is a Gram-positive microorganism commonly found in soil, which belongs to the *B. subtilis* group. Members of this genus have been described as producers of biotechnologically important compounds such as proteases, amylases, antibiotics and surfactants. The produced antimicrobial compounds include predominantly peptides that are either nonribosomally or ribosomally synthesized (Stein, 2005).

Lantibiotics are ribosomally synthesized as inactive prepropeptides which are post-translationally modified to their biologically active forms (Chatterjee *et al.*, 2005). The common structural feature of lantibiotics is the unusual amino acids lanthionine (Lan) and/or methyllanthionine (MeLan). Specific Ser and Thr residues are the precursors to enzymatic dehydration to yield 2,3-didehydroalanine (Dha) and 2,3-didehydrobutyrine (Dhb), respectively. Subsequently, Dha and Dhb form a thioether with thiol side chains of Cys via a Michael addition-type to yield Lan and MeLan, respectively (Willey and van der Donk, 2007). These post-translational modifications occur in the C-terminal region of the prepropeptide, while the unmodified N-terminal region (leader sequence) is proteolytically removed to yield the active lantibiotic (Chatterjee *et al.*, 2005).

Lantibiotics can be classified based on their maturation pathway and antimicrobial activity (Pag and Sahl, 2002; Willey and van der Donk, 2007). The release of *B. licheniformis* ATCC 14760 (isogenic to DSM13) genome (Rey *et al.*, 2004; Veith *et al.*, 2004) in 2004 allowed the identification of a putative lantibiotic gene cluster, which was recently associated with the lichenicidin production (Begley *et al.*, 2009; Dischinger *et al.*, 2009). Moreover and as established in chapter II, *B. licheniformis* I89 also produces such lantibiotic. According to a previously suggested classification (Willey and van der Donk,

2007), lichenicidin is a two-component lantibiotic, a subgroup of antibacterial class II lantibiotics. In two-component lantibiotics, two different structural genes (*lanA1* and *lanA*2) are expressed and subsequently modified by two different LanM proteins (LanM1 and LanM2). After post-translational modification both peptides are exported and their leader sequences removed by a single, multifunctional protein possessing an N-terminal protease domain designated LanT. Some of these compounds might undergo an additional N-terminal proteolytic step presumably by the action of an extracellular protease.

Generally, α- and β-peptides of two-component lantibiotics act synergistically to exert their full antibacterial activity. Presently, this group includes eight lantibiotics including haloduracin and lacticin 3147 (Ryan *et al.*, 1996; McClerren *et al.*, 2006; Lawton *et al.*, 2007). The lichenicidin complex is active against methicillin-resistant *Staphylococcus aureus* (MRSA) and *Listeria monocytogenes* (Begley *et al.*, 2009; Dischinger *et al.*, 2009). It is believed that two-component lantibiotics mechanism of action involves the interaction of both peptides through the binding to lipid II and formation of pores in the bacterial membrane (Oman and van der Donk, 2009b; Schneider and Sahl, 2010).

Apart from their antibacterial activity, other interesting bioactivities have recently been described for lantibiotics, e.g. type III lantibiotics possess antipain activity in a neuropathic pain mouse model (Meindl *et al.*, 2010). Consequently, during the last years, the evaluation of lantibiotic structure-activity relationships has increased, since the information retrieved can be further used in the rational design of new molecules with improved activity (Cortes *et al.*, 2009). Such studies, using site-directed mutagenesis, have already been conducted *in vivo* for lacticin 3147 peptides (Cotter *et al.*, 2006a) and *in vitro* for haloduracin (Cooper *et al.*, 2008). This approach was also useful for the revision of the proposed structure of the haloduracin β-peptide (Cooper *et al.*, 2008). Moreover, a saturation mutagenesis library of mersacidin was recently constructed yielding the production of 82 new compounds in which some of them demonstrated improved activity against Gram-positive pathogens (Appleyard *et al.*, 2009).

In this chapter, we report the first heterologous expression of a type-II two-component lantibiotic gene cluster in *E. coli* at the example of lichenicidin from *B. licheniformis* I89. Apart from immunity and regulation associated genes the functions of predicted open reading frames in the cluster were assigned. Finally, in *E. coli* a *trans* complementation system was established and new lichenicidin variants were generated, which allowed the assignment of essential residues for expression and antibiotic activity as well as the characterization of structural features previously proposed (Begley *et al.*, 2009). This is the

first report on lantibiotic production in Gram-negatives, opening new perspectives for the biotechnological studies of these compounds.

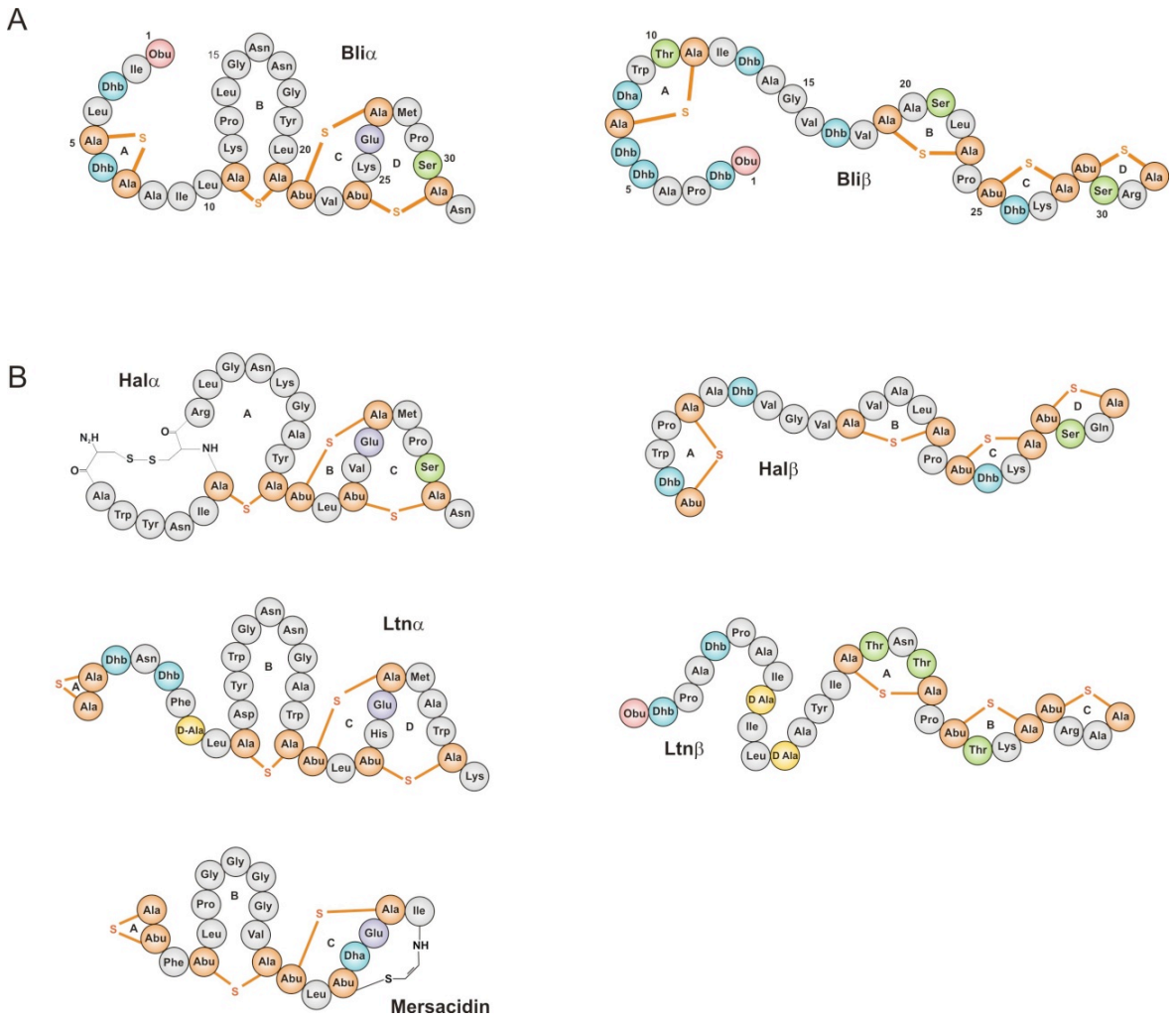

Figure 32: Proposed structures of Bliα and Bliβ peptides of the lichenicidin complex (A) and of the related lantibiotics haloduracin, lacticin 3147 and mersacidin (B). Non-dehydrated residues are represented in green and the highly conserved Glu residue in purple (Ala-S-Ala= lanthionine, Abu-S-Ala= methyllanthionine and OBu= 2-oxobutyryl).

4.2 Results

4.2.1 Synthesis of the two-component lantibiotics Bliα and Bliβ by *Bacillus licheniformis* I89

In the *B. licheniformis* ATCC 14760 genome a putative two-component lantibiotic gene cluster was identified (Rey *et al.*, 2004). The products of the structural genes *licA1* and *licA2* matched with the Bliα and Bliβ molecular masses assuming 7 and 12 dehydration reactions

of both propeptides. Both of these structural genes were also amplified from the *B. licheniformis* I89 bacterial genome. Recently, Begley *et al.* (2009) suggested that lichenicidin peptides (Bliα and Bliβ) production was encoded by this gene cluster (Figure 34A). However, it was Dischinger *et al.* (2009) experimentally proved such biosynthetic association. In this study (chapter II) we also demonstrated that *B. licheniformis* I89 strain was also able to produce the Bliα (M= 3249.7 Da; calculated M= 3248.58 Da) and the Bliβ (M= 3019.6 Da; calculated M= 3018.38 Da) peptides (Figure 33).

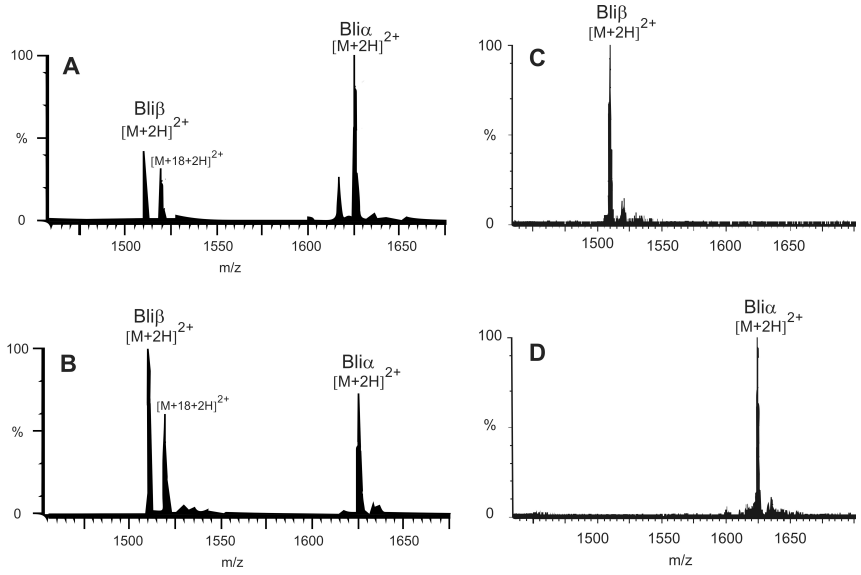

Figure 33: ESI-TOF mass spectra of Bliα (M=3249.54 Da) and Bliβ (M=3019.38 Da) peptides detected in *B. licheniformis* I89 supernatant (A) and cell (B) extracts. *licA1* and *licA2* gene inactivated mutants in Lic5ΔA1 and Lic5ΔA2 resulted in the production of only Bliβ (C) and Bliα (D), respectively.

4.2.2 Heterologous expression of lichenicidin in *Escherichia coli*

B. licheniformis I89 exhibited low transformation efficiencies, probably due to class-I restriction modification systems previously identified in *B. licheniformis* DSM13 (Rey *et al.*, 2004; Veith *et al.*, 2004). This is a severe impediment for the molecular biology procedures necessary for gene function analysis and also for rapid access to mutant strains. Thus, heterologous expression of lichenicidin was attempted in the Gram-negative host *Escherichia coli*. To access the complete gene cluster in a vector, a fosmid library of *B. licheniformis* I89 genomic DNA was constructed in *E. coli* EPI300 and screened with a DIG-labelled DNA probe encoding the lichenicidin structural genes *licA1* and *licA2*. Five positive clones (Lic5, Lic7, Lic8, Lic10 and Lic45) were identified, containing the *lic* gene cluster (approximately 14 Kb) (Figure 34A). All the clones inhibited the growth of *M. luteus*

and therefore one clone (Lic5) containing the fosmid pLic5 (Figure 34B) was selected as lichenicidin heterologous expression system.

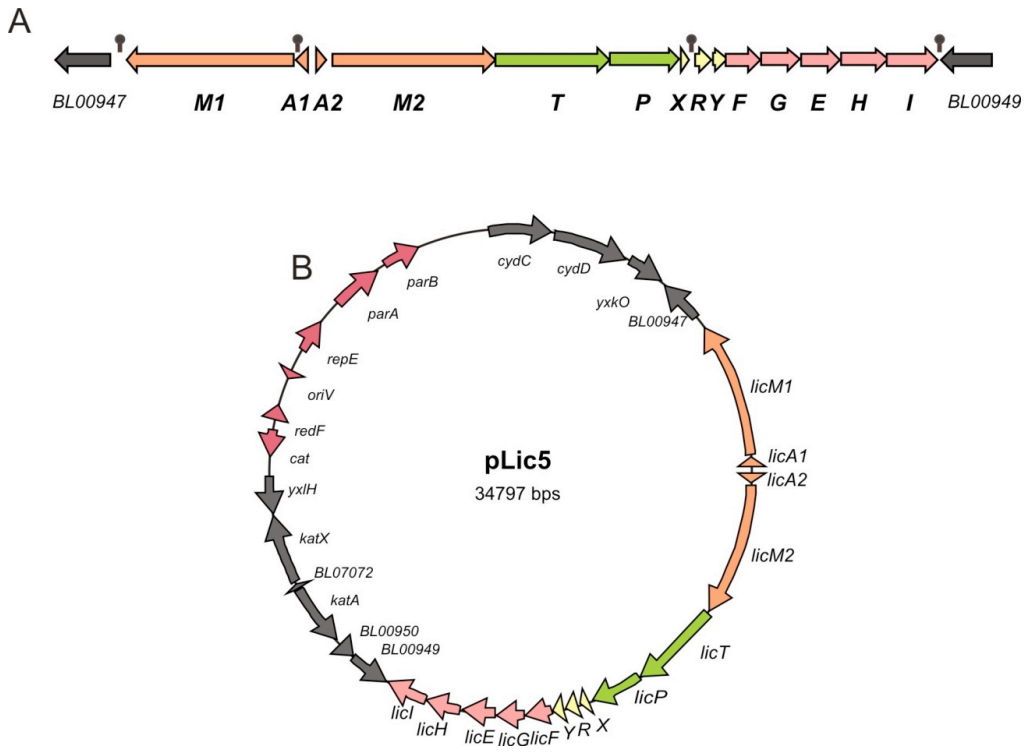

Figure 34: Organization of the lichenicidin biosynthetic gene cluster, according to the genome annotation for *Bacillus licheniformis* ATCC 14760 (A); the black circles correspond to deduced Rho-dependent terminators. Representation of the pLic5 fosmid map, containing the lichenicidin biosynthetic gene cluster (B).

Lichenicidin production by Lic5 was evaluated along 36 hours in liquid cultures. LC-ESI-MS analysis of both cell isopropanol washes and supernatant extracts after 24 hours of growth revealed the presence of peptides with molecular masses corresponding to Bliα and Bliβ. The exiting observation of successful heterologous expression of the *lic* cluster in a Gram-negative organism required closer consideration and experimental proof. Hence, detection of Bliα and Bliβ observed in *E. coli* supernatants could either be explained by bacterial lysis of the producing strain or by active export through the outer membrane. Since growth behaviour of *E. coli* carrying the *lic* gene cluster was not conspicuous the latter hypothesis was investigated, analyzing the influence of TolC, an outer membrane protein commonly involved in the export of toxins on lichenicidin production. Therefore, *E. coli* BW25113 possessing a deletion of the *tolC* gene was transformed with the fosmid pLic5 carrying the *lic* cluster. The resulting *E. coli* BW25113Δ*tolC*:*kan*-pLic5 strain did not show antibacterial activity against the indicator strain *M. luteus* (Figure 35) and the

molecular masses of Bliα and Bliβ were not detected by LC-ESI-MS analysis of supernatant extracts. *Trans* complementation of this strain with *tolC* resulted in restoration of antibacterial activity (Figure 35). This was further corroborated by detection of characteristic masses of Bliα and Bliβ by means of HPLC-ESI-MS from supernatant extracts.

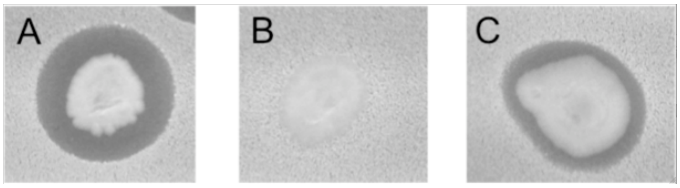

Figure 35: Colony bioassay of *E. coli* BW25113 (A) and BW25113Δ*tolC:kan* (B) possessing the fosmid pLic5. *trans*-complementation of *E. coli* BW25113Δ*tolC:kan* strain with *tolC* gene resulted in the reestablishment of activity (C).

4.2.3 Mutagenesis of the lichenicidin biosynthetic cluster

The *lic* biosynthetic gene cluster (Figure 34A) is composed of 14 open reading frames (ORFs). Expression of these genes in *E. coli* Lic5 further permitted the construction of gene-inactivated mutants using the λ RED recombinase system (Datsenko and Wanner, 2000; Gust *et al.*, 2003). To that end, the fosmid pLic5 was introduced into BW25113 cells containing the RED recombinase expression plasmid pKD20 (Datsenko and Wanner, 2000). The transformed strain was used as a platform for the inactivation of all ORFs of the *lic* cluster, except those presumably associated with self-immunity. In total, nine fosmids were constructed, each of them containing the deletion of one gene in BW25113 cells and transformed into EPI300 cells (Table 6). Overall, the expression of eight ORFs was found to be critical for lichenicidin production (Figure 36A). Only the Lic5Δ*X* knockout mutant retained visible antibacterial activity against the indicator strain *M. luteus* (Figure 36A).

According to previous studies on two-component lantibiotics, it was expected that the single production of either Bliα or Bliβ would result in significantly reduced antibacterial activity. Consequently, the loss of activity by mutational inactivation of *lic* genes could result from the absence of production of both or only one of the lichenicidin peptides. If the second assumption was correct, antibacterial activity should be restored if the complementing peptide could be externally supplied by cross-feeding. Indeed, this was observed when the two strains missing the respective intact structural genes (Lic5Δ*A1* and Lic5ΔA2) were bioassayed as neighbouring colonies (Figure 36B). Moreover, no *M. luteus* inhibition was observed when Lic5Δ*A1* mutant was inoculated next to Lic5Δ*A1*, or Lic5ΔA2 next to Lic5ΔA2. LC-ESI-MS analysis of both clones revealed that Lic5Δ*A1* synthesized

exclusively the Bliβ peptide and Lic5Δ*A2* synthesized exclusively the Bliα peptide (Figure 33C/D). These results showed that inhibition of *M. luteus* was a consequence of the synergistic activity of both peptides. Therefore, Lic5Δ*A1* and Lic5Δ*A2* were used in cross-feeding agar-diffusion assays with the other knockout mutants. Full restoration of antibacterial activity was observed between the following combinations of knockout mutant pairs: Lic5Δ*A1* with Lic5Δ*M2*, Lic5Δ*P* and Lic5Δ*R*; Lic5Δ*A2* with Lic5Δ*M1* and Lic5Δ*Y* (Figure 36C). These results were predictive of peptide production in each knockout mutant and were unambiguously confirmed by LC-ESI-MS analysis (Table 6).

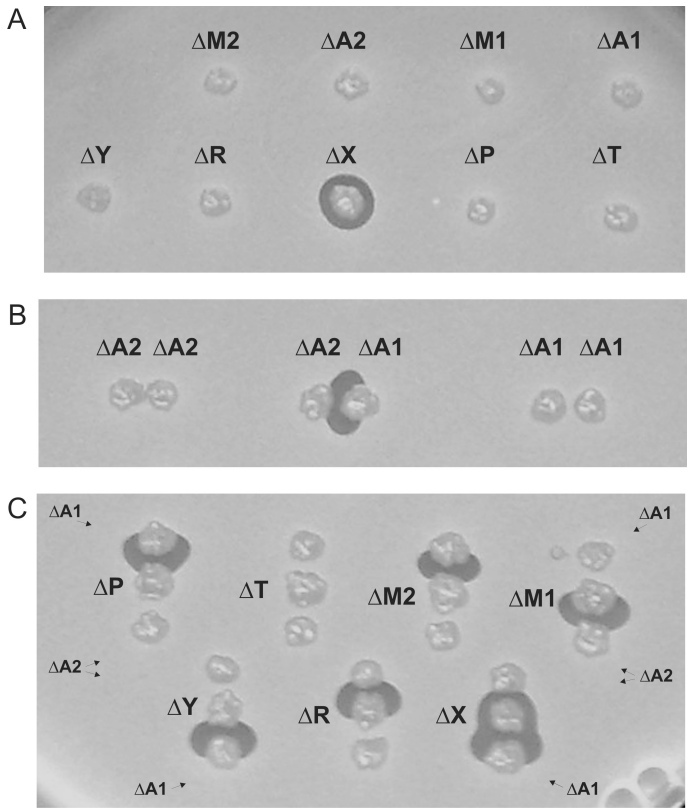

Figure 36: Agar diffusion assay for the assessment of lichenicidin production by knockout mutants of the *lic* gene cluster with *M. luteus* as the indicator strain. Lic5 gene inactivation mutants are represented by Δ*A1*, Δ*A2*, Δ*M1*, Δ*M2*, Δ*T*, Δ*P*, Δ*X*, Δ*R* and Δ*Y*, according with the inactivated gene. (A) Antibacterial activity exhibited by all knockout mutants produced in this study. (B) Synergetic activity of peptides Bliα and Bliβ, produced by Lic5Δ*A2* and Lic5Δ*A1*, respectively. (C) Restored lichenicidin activity of the knockout mutants upon interaction with Bliβ and Bliα peptides produced by Lic5Δ*A1* and Lic5Δ*A2*, respectively.

LC-ESI-MS analysis of the mutants obtained from inactivation of dehydratase-cyclases Lic5Δ*M1* and Lic5Δ*M2*, showed the same results as those obtained with the mutants of the structural genes Lic5Δ*A1* and Lic5Δ*A2*, respectively. Molecular masses corresponding to propetides or prepropeptides were not detected.

Sequence homology studies identified LicT from the *B. licheniformis* I89 *lic* cluster as a member of the ABC transporter family with an integrated protease domain, presumably responsible for removal of the leader sequences of Bliα and Bliβ during substrate translocation. In *E. coli*, the inactivation of *licT* (Lic5Δ*T*) resulted in the loss of visible antibacterial activity, which was not restored by interaction with Lic5Δ*A1* or Lic5Δ*A2*. Accordingly, molecular masses of Bliα and Bliβ peptides were not identified in the Lic5Δ*T* supernatant extracts by LC-ESI-MS. However, when isopropanol washes of Lic5Δ*T* cells were analyzed by LC-ESI-MS a peptide mass corresponding to fully processed Bliβ was identified, albeit in extremely low concentration. It is likely that isopropanol treatment of Lic5Δ*T* cells released Bliβ propeptide to interact with the protease LicP (active in this mutant), thus yielding correctly processed Bliβ peptide without the leader sequence.

Table 6: List of Lic5 gene inactivation mutants constructed in this study and the assigned function of the deleted gene. The restoration of antibacterial activity observed in cross-feeding experiments is listed, considering Lic5*ΔA1* and Lic5Δ*A2* as exclusive producers of Bliβ and Bliα peptides, respectively. A comparison between the expected and observed lichenicidin peptide molecular masses of each mutant was included. (ND = none of the peptides detected). *- The Bliβ peptide was only detected in the cell-wall washes.

***E. coli* strains**	**Gene deleted**	**Restore of activity**	**Peptide masses (Da)**	
			Expected molecular mass	**Observed molecular mass**
Lic5Δ*M1*	*licM1*, modifying enzyme of LicA1 peptide	LicΔ*A2*	3019.36	3019.60
Lic5Δ*A1*	*licA1*, Bliα peptide structural gene	LicΔ*A2*	3019.36	3019.60
Lic5Δ*A2*	*licM2*, modifying enzyme of LicA2 peptide	LicΔ*A1*	3249.54	3249.50
Lic5Δ*M2*	*licM2*, Bliβ peptide structural gene	LicΔ*A1*	3249.54	3249.50
Lic5Δ*T*	liciT, transporter and leader sequence proteolysis of LicA1 and LicA2 peptides	ND	ND	3019.60*
Lic5Δ*P*	*licP*, putative peptidase responsible for Bliβ N-terminal processing	LicΔ*A1*	3249.54	3249.50
Lic5Δ*X*	*licX*, unknown function	LicΔ*A1*	3019.36 and 3249.54	3019.60 and 3249.50
Lic5Δ*R*	*licR*, putative lantibiotic regulator	LicΔ*A2*	3019.36	3019.60
Lic5Δ*Y*	*licY*, unknown function	LicΔ*A1*	3249.54	3249.50

The *licX* gene encodes a small-uncharacterized hypothetical protein with significant homology to other uncharacterized *Bacillus* sp. proteins. The absence of conserved core motifs in this protein makes it difficult to predict its function. When *licX* was inactivated, the antibacterial activity of Lic5Δ*X* was not affected and both peptides Bliα and Bliβ were identified in LC-ESI-MS analysis, indicating that LicX does not play an essential role in lichenicidin production.

Upstream of *licX*, two other genes, *licR* and *licY*, were identified. NCBI Conserved Domain Searches of the *licR* sequence revealed a high homology to helix–turn–helix XRE family-like proteins. After its deletion only Bliβ was identified by LC-ESI-MS analysis of Lic5Δ*R* extracts. This result demonstrates that LicR might have an essential regulatory function in the biosynthesis of mature LicA2 peptide. Finally, the Lic5Δ*Y* mutant was identified as the only producer of the Bliα peptide. This proved an essential involvement of LicY in the biosynthesis of Bliβ but not in the biosynthesis of Bliα. Three transmembrane helices identified in LicY point to a likely role as a membrane protein.

4.2.4 LicP, a protease involved in Bliβ biosynthesis

Bliα and Bliβ prepropeptides both have a common proteolytic site for LicT (C-terminal of the GlyGly-motif). The molecular mass of the processed Bliβ peptide affords additional proteolysis of an N-terminal hexapeptide of the Bliβ' peptide (corresponding to H_2N-NDVNPE-Bliβ). Such second proteolysis step has been described for other two-component lantibiotics, e.g. Halβ, Plwβ, $CylL_L$ and $CylL_S$ (Booth *et al.*, 1996; Holo *et al.*, 2001; McClerren *et al.*, 2006). In the *lic* gene cluster, *licP* encodes a putative uncharacterized serine protease homologous to CylA (38% identity) from *Enterococcus faecalis* ($CylL_L$ and $CylL_S$ producer) and to BH1491 uncharacterized protease (30% identity) from *B. halodurans* C-125 (Halβ producer). Therefore, it was considered that LicP could be the protease post-operative to LicT responsible for the removal of the hexapeptide NDVNPE from Bliβ'. In fact, when *licP* was deleted only the production of Bliβ was negatively affected. The *E. coli* Lic5Δ*P* mutant lost antibacterial activity, which could be restored upon cross feeding with a mutant delivering correctly processed Bliβ peptide e.g. mutant Lic5Δ*A1* (Figure 36C). Hence, in Lic5Δ*P* isopropanol washes of cells and supernatant extracts only the molecular mass of the Bliα was identified. Molecular masses corresponding to the N-terminally untrimmed Bliβ peptide (calculated M=3686.65 Da) or those of its derivatives could not be identified. However, when *E. coli* Lic5Δ*P* cell and supernatant extracts where bioassayed next to filter-sterilized supernatants of an *E. coli* strain solely expressing LicP (BL21 *licP*$^+$), the reestablishment of antibacterial activity was observed (Figure 37). This indicated that fully active Bliβ was produced *in situ* and could act synergistically with the Bliα peptide.

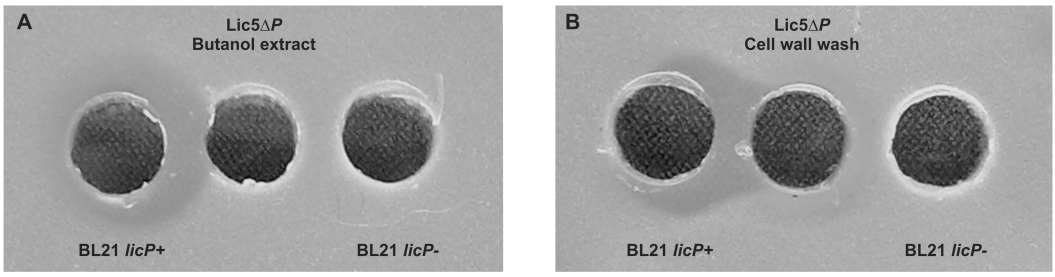

Figure 37: Cell isopropanol washes and supernatant butanol extracts of the knockout mutant Lic5Δ*P* were assayed side by side with the cell-free supernatants of BL21licP+ and BL21licP-. BL21licP+ corresponds to the *E. coli* strain expressing exclusively LicP protein. BL21licP- corresponds to the *E. coli* control strain harboring the empty pET24-a(+) vector. Inhibition of *M. luteus* is observed exclusively when Lic5Δ*P* extracts are in contact with the supernatant of BL21licP+ strain.

4.2.5 A complementation system for expression of lichenicidin peptide derivatives in *E. coli*

The construction of a system enabling generation of lichenicidin variants was developed, due to its usefulness for both structure-activity relationship (SAR) analysis and structure elucidation purposes. To that end, *E. coli* BL21Gold® producing exclusively one of the proprepeptides were obtained by transformation of these cells with pLic5Δ*A1* or pLic5Δ*A2* fosmids, generating BLic5Δ*A1* and BLic5Δ*A2* strains, respectively. These strains were complemented with the respective deleted genes (*licA1* and *licA2*) cloned into the expression vector pET-24a(+). The resulting transformants BLic5Δ*A1*$^+$ (BLic5Δ*A1*+p*licA1*) and BLic5Δ*A2*$^+$ (BLic5Δ*A2*+p*licA2*) were able to inhibit the indicator strain and the molecular masses of both Bliα and Bliβ by LC-ESI-MS were successfully identified. Therefore, this system was further exploited by PCR site-directed mutagenesis of p*licA1* and p*licA2* in order to obtain *E. coli* strains producing Bliα and Bliβ variants. During this procedure, only one of the lichenicidin peptides was mutated at each time, facilitating an exact assignment of the impact of the introduced alteration also in production and/or bioactivity. It was attempted to generate 32 lichenicidin variants, which molecular masses are summarized in Table 7.

In this study a systematic Ala scan was performed for all Cys, Ser and Thr residues of Bliα and Bliβ. Especially C-terminal substitutions of residues BliαThr24, BliβThr25, BliβCys28 and BliβThr29A did not yield the corresponding mutant peptide (HPLC-ESI-MS control) and consequently no bioactivity was observed (Figure 38). All other lichenicidin variants were synthesized although to a different extent. Some of the peptides variants were produced

in low amounts and therefore were not used in bioactivity assays (Figure 38). Peptides that were produced in amounts comparable to the wild type (HPLC-ESI-MS control) were correlated to bioactivity in agar diffusion tests. Overall, the changes introduced at the N-terminus of Bliα and Bliβ seem to be less prejudicial for lichenicidin bioactivity and/or for peptide production than those at the C-terminus (Figure 38).

Table 7: List of molecular masses obtained for alanine-scan mutants. Monoisotopic molecular masses detected by high-resolving ESI-TOF-MS in supernatant extracts of Bliα and Bliβ alanine-scan mutants. "Expected 1" represent the exact masses calculated based in previously proposed structures (Begley *et al.*, 2009). "Expected 2" represents the lichenicidin exact masses accordingly with the structure proposed in this study. ND represents bioengineered peptides that were not detected by LC-ESI-MS analysis.

Bliα peptides	Molecular mass (Da)		
	Expected 1	Expected 2	Observed
Bliα	3248.53	3249.52	3249.54
BliαT1A	3236.53	3236.53	3236.56
BliαT3A	3236.53	3237.52	3235.56
BliαS5A	3250.55	3251.53	3251.58
BliαT6A	3236.53	3237.52	3237.54
BliαC7A	3216.56	3217.54	3217.58
BliαS11A	3250.55	3251.53	3251.56
BliαC21A	3216.56	3217.54	3217.58
BliαT22A	3236.53	3237.52	3235.56
BliαT24A	3236.53	3237.52	ND
BliαE26A	3190.53	3191.51	3191.54
BliαC27A	3216.56	3217.54	3217.59
BliαS30A	3232.54	3233.52	3233.58
BliαC31A	3216.56	3217.54	3217.59

Bliβ peptides	Molecular mass (Da)		
	Expected 1	Expected 2	Observed
Bliβ	3018.38	3019.36	3019.38
BliβT1A	3006.38	3006.38	3006.42
BliβT2A	3006.38	3007.36	3007.40
BliβT5A	3006.38	3007.36	3007.40
BliβT6A	3006.38	3007.36	3007.40
BliβS7A	3020.39	3021.38	3021.42
BliβS8A	3020.39	3021.38	3021.42
BliβT10A	3006.38	2989.35	2989.20
BliβC11A	2986.40	2987.39	2987.42
BliβT13A	3006.38	3007.36	3007.40
BliβT17A	3006.38	3007.36	3007.40
BliβS19A	3020.39	3021.38	3021.42
BliβS21A	3002.38	3003.37	3003.32
BliβC23A	2986.40	2987.39	2987.48
BliβT25A	3006.38	3007.36	ND
BliβT26A	2988.37	3007.36	3007.40
BliβC28A	2986.40	2987.39	ND
BliβT29A	3006.38	3007.36	ND
BliβS30A	3002.38	3003.37	3003.40
BliβC32A	2986.40	2987.39	2987.60

Homology studies of mersacidin-like two-component lantibiotics revealed the presence of a highly conserved glutamate (Glu17; Figure 32). Its substitution in mersacidin, Halα and Ltcα completely abolished antibacterial activity (Cotter *et al.*, 2006a; Cooper *et al.*, 2008; Appleyard *et al.*, 2009). For mersacidin it was proved that Glu17 is essential for binding to the lipid II target (Brotz *et al.*, 1998; Hsu *et al.*, 2003; Szekat *et al.*, 2003). In the case of lichenicidin Bliα, the substitution of homologous Glu26 by Ala yielded the expected peptide (M= 3191.54 Da), however antibacterial activity of BliαE26A was completely abolished.

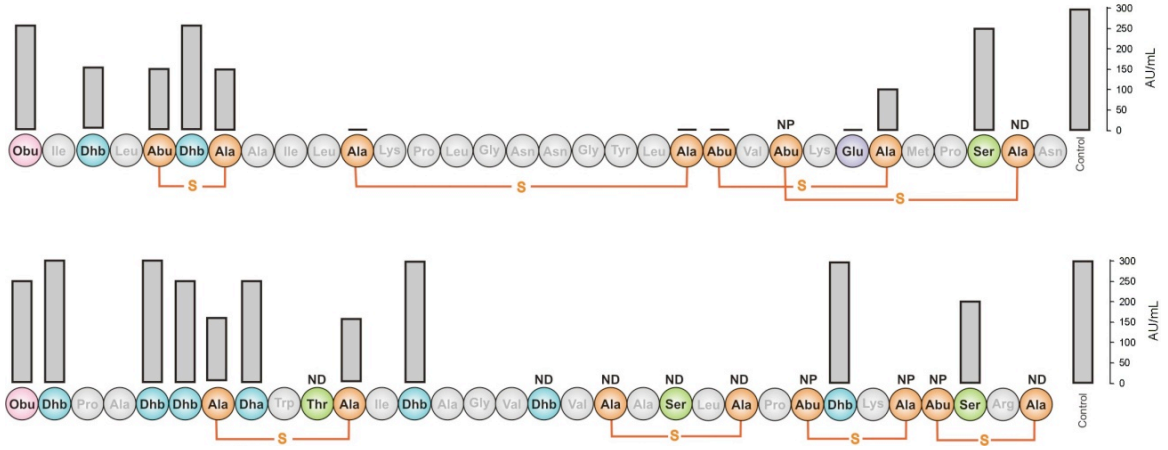

Figure 38: Supernatant extracts activity (AU.ml^{-1}) of Bliα (A) and Bliβ (B) alanine-scan mutants against *M. luteus*. Control strains represent the mutants BLicΔ*A1* and BLicΔA2, complemented with p*licA1* (A) and p*licA2* (B). The primary amino acid sequence and thioether rings of Bliα and Bliβ are represented underneath the bioactivity results, accordingly with the results obtained in this study (OBu = 2-oxobutyryl). Non-producer (NP) strains or low-producer (LA) strains of mutated peptides were not integrated in the bioassay.

4.2.6 Localization of the N-terminal lanthionine bridges of Bliα and Bliβ

The determined exact molecular masses of Bliα and Bliβ peptides (M= 3249.54 Da and M= 3019.6 Da, respectively) differ from the calculated masses by +1 Da, suggesting that a 2-oxobutyryl residue could be present at the N-terminus of both peptides. This hypothesis was also supported by mutant peptides of Bliα and Bliβ possessing an N-terminal Thr→Ala replacement thus retaining a conventional N-terminus (BliαT1A: M= 3236.52 Da and BliβT1A: M= 3006.36 Da) and bioactivity. Hence, establishment of N-terminal A-rings of Bliα and Bliβ between Dhb1 and Cys7 (Bliα) and Dhb1 and Cys11 (Bliβ) seemed less likely, contrary to data previously proposed by (Begley *et al.*, 2009). MS/MS analysis of the Bliα peptide from *B. licheniformis* I89 (Figure 39) and *E. coli* BLic5 supernatants showed the presence of the N-terminal ions a_3 and b_3, excluding the possibility of A-ring formation between Cys7 and Dhb1 or Dhb3. Instead, either Dha5 or Dhb6 were candidates for A-ring formation with Cys7. This question was further investigated by MS/MS experiments of Bliα peptides containing an Ala5 (BliαS5A mutant) or Thr6 (BliαT6A mutant) (Figure 40).

The identification of the y_{26} ion for BliαS5A but not for BliαT6A supports a Bliα structure with an A-ring between Dha5 and Cys7 (Figure 32A, Figure 40). The MS/MS spectra of the Bliβ peptide from *B. licheniformis* I89 (Figure 39) and *E. coli* BLic5 supernatant permitted the identification of a series of fragment ions (a_2, b_2, a_3, b_3, b_5, b_6, y_{27} and y_{26}), suggesting that only Dha7 or Dhb8 were likely to be involved in A-ring formation with Cys11. The analysis of the bioactivity of the peptides possessing an Ala residue instead of Dhb2, Dhb5, Dhb6,

Dha7 and Dha8 suggests that Dha7 should be the residue reacting with Cys11 of Bliβ peptide (Figure 32A and Figure 38).

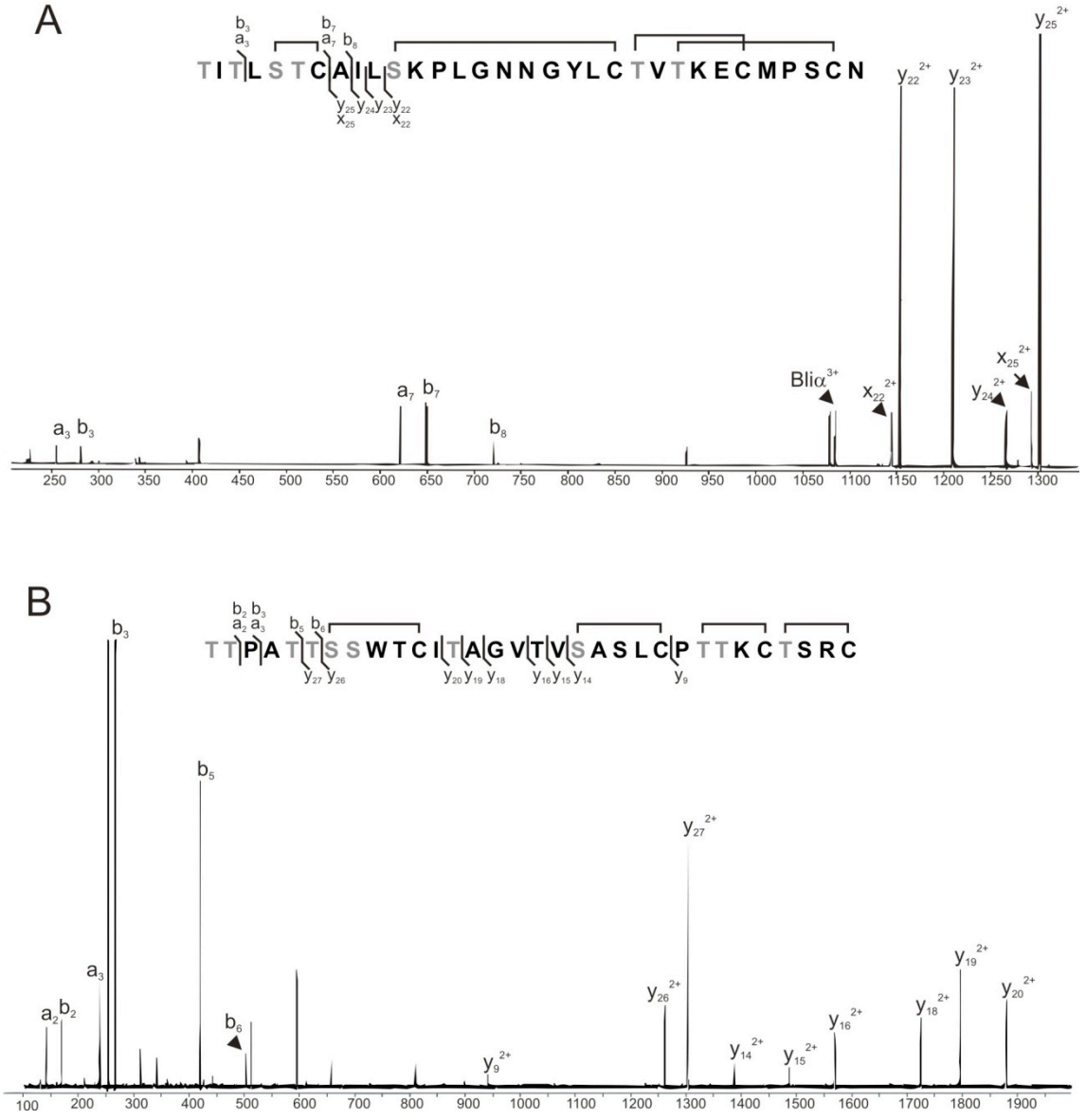

Figure 39: ESI-MS/MS spectra of Bliα (A) and Bliβ (B) purified from the *B. licheniformis* I89 supernatant and assigned fragmentation pattern of lichenicidin peptides.

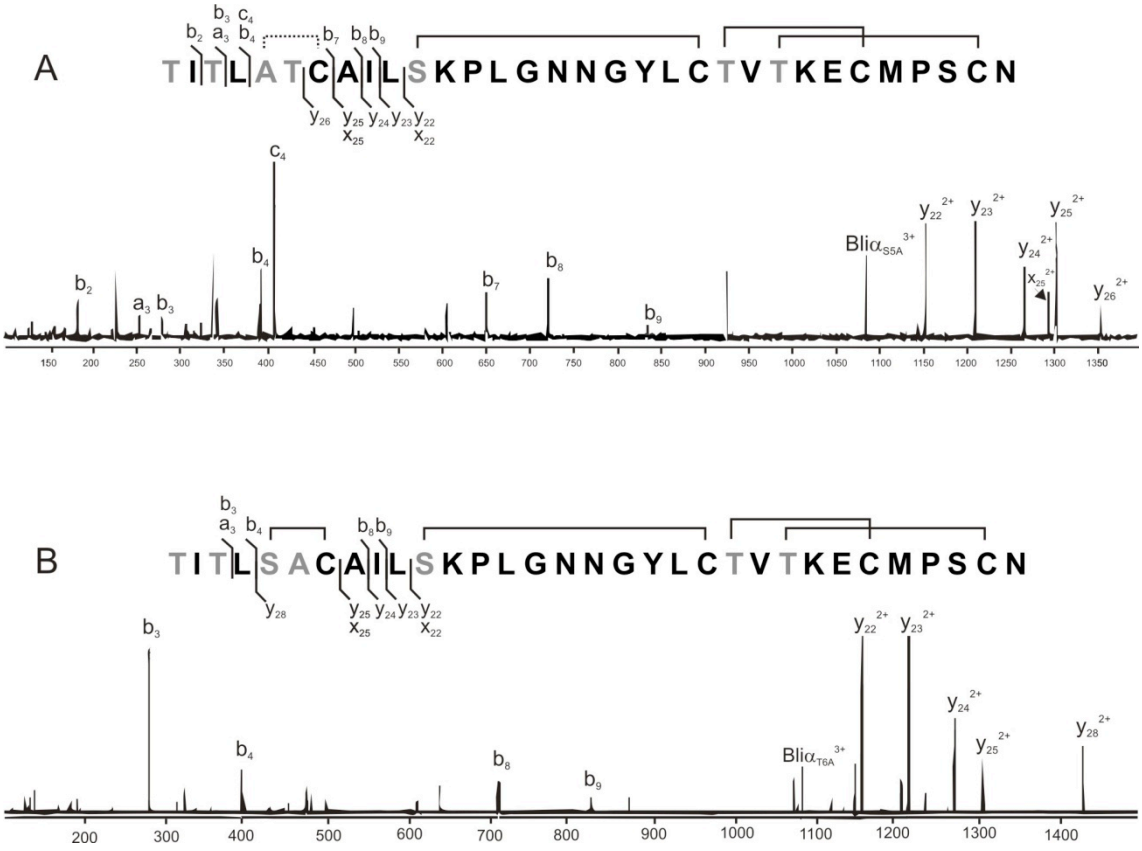

Figure 40: ESI-MS/MS spectra (Agilent Triple Quad 6460) of BliαS5A (A) and BliαT6A (B) peptide mutants.

4.2.7 Localization of 2,3-didehydro amino acids in the structures of Bliα and Bliβ peptides

The found molecular masses of Bliα and Bliβ peptides are consistent with the post-translational dehydration of seven and twelve Ser/Thr residues, respectively. Therefore, one and three Ser/Thr should escape the action of the dehydratase-cyclases LicM1 and LicM2, respectively. These residues were proposed to be Ser30 of Bliα and Ser21, Thr26 and Ser30 of Bliβ (Begley *et al.*, 2009). To confirm this assumption experimentally, these four residues were mutated to Ala. Analysis of the BliαS30A mutant revealed the presence of a seven-fold dehydrated peptide (M= 3233.58 Da). Therefore, Ser30 from Bliα escapes dehydratation. The same analysis for Bliβ mutants BliβS21A and BliβS30A revealed products with molecular masses corresponding to the expected twelve dehydratations (M= 3003.40 Da). Therefore, LicM2 does not modify Ser21 and Ser30. However, the peptide produced by BliβT26A mutant (M= 3007.40 Da) was consistent with the occurrence of only eleven, instead of the proposed twelve dehydratations (calculated M= 2989.37 Da) (Begley *et al.*,

2009). The substitution of Thr10→Ala resulted in the synthesis of a twelve-fold dehydrated peptide (M= 2989.72 Da; Figure 41 and Table 7). Thus, Thr10 is the candidate amino acid escaping LicM2 dehydration and this finding was also included in the Bliβ structure (Figure 32A).

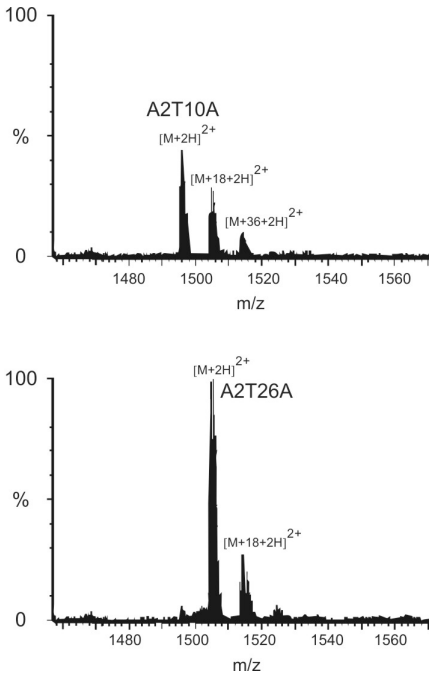

Figure 41: ESI-TOF-mass spectra of mutants LicA2T10A (M=2989.20 Da) and LicA2T26A (M=3007.40 Da), showing the modification of Thr26 by LicM2. Peptides corresponding to different stages of dehydration were also identified.

4.3 Discussion

In the light of recent developments in the field of lantibiotic biosynthesis, the role of lantibiotics for use as antimicrobials is newly weighted. This is supported by observations that when compared to other commonly used antibacterials the development of resistance is believed to be extremely low. Lichenicidin is a two-component lantibiotic (Bliα and Bliβ) produced by *B. licheniformis*. Recently, two independent studies assigned its production to a biosynthetic gene cluster encoded in *B. licheniformis* ATCC 14760 and DSM13 (Begley *et al.*, 2009; Dischinger *et al.*, 2009). Although not recognized at that time, Bliα production was also reported by Mendo and coworkers in *B. licheniformis* I89 supernatant extracts (Mendo *et al.*, 2004). Herein, lichenicidin peptides Bliα and Bliβ were detected in cell washes and supernatant extracts of this strain. Thus, in the I89 strain this lantibiotic is not exclusively cell wall-associated as it has been described for other strains of this specie (Begley *et al.*, 2009; Dischinger *et al.*, 2009).

B. licheniformis l89 was not amenable to transformation and therefore heterologous expression of lichenicidin was attempted in *E. coli*. This Gram-negative organism is a preferred heterologous expression host with respect to genetic manipulation, handling cost and time considerations. The presence of the fosmid p*Lic5* harboring the complete lichenicidin gene cluster in *E. coli* (Lic5) resulted in a strain able to inhibit *M. luteus* growth. Characteristic molecular masses of fully modified Bliα and Bliβ peptides were detected by LC-ESI-MS in *E. coli* Lic5 cell washes (isopropanol) and supernatant extracts. In the past, the heterologous production of lantibiotics in *E. coli* hosts was unknown. Recently, first steps into this direction where undertaken by the co-expression of proprepeptide and the corresponding modifying enzyme in *E. coli*, which achieved establishment of the fully dehydrated lantibiotic BovHJ50 (Lin *et al.*, 2010). However, its biologically active form was only obtained after *in vitro* incubation with a specific protease (BovT150) to remove the leader sequence. In contrast, herein we obtained a full reconstitution of the *in vivo* synthesis of a lantibiotic in a Gram-negative host. Moreover, heterologous expression of the *lic* cluster in a *tolC*-deficient *E. coli* strain revealed the absence of Bliα and Bliβ peptides in supernatant extracts. This supported the assumption that TolC and/or TolC-related proteins are likely candidates for the transport of Bliα and Bliβ through the outer membrane, which was further corroborated by restoration of lichenicidin activity upon *tolC trans*-complementation.

The lichenicidin gene cluster consists of 14 ORFs and in the past, only the expression of *licM1* and *licM2* genes was associated with lichenicidin production (Dischinger *et al.*, 2009). Therefore, λ RED recombinase technology was employed to inactivate all *lic* genes in the p*Lic5* fosmid, except those putatively related with immunity. Our results showed that *licA1* and *licA2* encode the lichenicidin proprepeptides, which are post-translationally modified by bifunctional dehydratases-cyclases LicM1 and LicM2, respectively. In the absence of LicT *(E. coli* Lic5Δ*T)*, it was not possible to detect the fully processed Bliα and Bliβ peptides in supernatant extracts, indicating that LicT protein plays an essential role in the maturation of both peptides. LicT protein is a member of the ABC transporter family with an integrated protease domain, apparently responsible for the removal of the leader sequences of Bliα and Bliβ during their transport. *E. coli* proteases and toxins are often exported directly from the cytoplasm to the supernatant by a *sec*-independent type I secretion system (T1S). T1S affords the presence of an ABC transporter, a membrane fusion protein (MFP) and an outer membrane protein (OMP; e.g. TolC) (Kostakioti *et al.*, 2005). Considering this system, it could be hypothesized that LicT located in the inner membrane is able to closely interact with MFP and TolC (or TolC-related protein) of *E. coli*, forming a T1S-complex responsible for leader peptide removal and export of Bliα and Bliβ. It is also

conceivable, that LicT removes the leader peptides and transports Bliα and Bliβ to the periplasmatic space followed by TolC-mediated export without any interaction between LicT and TolC. Alternatively, Bliα and Bliβ could be exported by inner and outer membrane transporters provided by the *E. coli* host. Then LicT would only contribute a proteolytic function required for leader peptide removal. Nevertheless, these hypotheses have to be further evaluated experimentally in order to establish a comprehensive model for lantibiotics secretion in Gram-negative hosts.

LicP is a serine protease homologous to the extracellular protease CylA (38% identity) which is responsible for the N-terminal trimming of cytolysin peptides $CylL_L$ and $CylL_S$, after their export (Booth *et al.*, 1996). In this study, LicP was also shown to be a protease involved exclusively in Bliβ maturation, by excising the N-terminal hexapeptide from NDVNPE-Bliβ. Moreover, LicP was present in the supernatants of an *E. coli* strain expressing exclusively this protease. The analogous function in Halβ peptide maturation was previously proved *in vitro* in studies performed with an undetermined protease present in supernatants of *B. halodurans* C-125 (Cooper *et al.*, 2008). The detection of Bliβ peptide in LicΔ*T* isopropanol cell washes, albeit in extremely low amounts, suggests that LicP proteolysis also can occur in the presence of the full leader sequence.

The LicX sequence is similar to other uncharacterized hypothetical proteins of the *Bacillus* genus and no conserved motifs could be identified, making it difficult to predict its function. Yet, we could establish that LicX is not involved in the Bliα and Bliβ biosynthesis or its function could be complemented when using *E. coli* as the heterologous host. LicR is a predicted DNA-binding protein with high similarity (60 %) to other transcriptional regulators from *Bacillus* genus. The *licR* gene inactivation proved the exclusive involvement of LicR in Bliα production, most probably through the induction of *licA1* transcription. Likewise, disruption of a similar gene, *mrsR1* present in the mersacidin biosynthetic cluster strongly decreased its proprepeptide transcription rate resulting in the absence of MrsA production (Guder *et al.*, 2002; Schmitz *et al.*, 2006). Since Bliα and MrsA peptides are highly homologous (Figure 32) and are produced by *Bacillus* species, it seems possible that their regulation mechanisms have evolved from a common ancestor. The LicY protein has been found essential for production of the Bliβ peptide, but not for production of Bliα. LicY does not display significant similarities to other proteins available in databases, making it impossible to assign a functional role to this protein. Like CylR1 of the cytolysins (Coburn and Gilmore, 2003) the LicY secondary structure is predicted to contain three transmembrane helices and a function as a membrane-bound protein seems likely. CylR1 and CylR2 have been described as a two-component regulatory system, which represses the expression of cytolysin genes through a quorum-sensing mechanism (Haas *et al.*, 2002).

Nevertheless, it is difficult to apply this model to the regulation of Bliβ expression, since it was not possible to identify a gene encoding DNA-binding protein within the *lic* biosynthetic cluster, which could be associated with Bliβ production. In this study, we assigned for the first time the association of two putative distinct regulatory elements to the production of one specific single peptide, Bliα or Bliβ, of the lichenicidin two-component system. We hypothesize, that analogous mechanisms of regulation are involved in the biosynthesis of other two-component lantibiotics, e.g. of haloduracin. In fact, the proteins BH0460 and BH0459 encoded downstream of *hal* biosynthetic gene cluster show high homology to LicR (60 %) and LicY (61 %), respectively. Further studies examining gene cluster transcription and protein interaction will shed light on the assignment of the exact role of LicR and LicY on lichenicidin production.

The successful heterologous expression of the *lic* gene cluster in *E. coli* facilitated the establishment of a system for the expression of lichenicidin variants. The peptides resulting from Ala substitutions and subsequent MS/MS analysis (of the wild type or mutated peptides) allowed the revision of some structural features of both Bliα and Bliβ previously proposed by (Begley *et al.*, 2009). Compared to the structurally related two-component lantibiotics haloduracin and lacticin, the high number of serines and threonines in lichenicidin propeptide sequences, particularly at the N-termini, made a clear assignment of dehydrated residues difficult. The Ala-scan performed for all Ser- and Thr-containing positions confirmed that Ser30 escapes dehydration in Bliα and likewise, Thr10, Ser21 and Ser30 remain unmodified in the Bliβ peptide. Herein, it was established that LicM2 dehydrates Thr26 of Bliβ, similarly to the homologous residue in Halβ (Dhb18), but in contrast to Lctβ (Thr23). The exact molecular masses obtained for Bliα and Bliβ exceed those of previously determined peptides by +1 Da, indicating that after proteolysis, a spontaneous deamination of Dhb1 to Obu1 occurs in both Bliα and Bliβ, as described for Pep5 and Lctβ peptides (Ryan *et al.*, 1999). This is clearly supported by mass spectrometry of peptides BliαT1A and BliβT1A suggesting that Thr1 of both peptides is not involved in A ring formation. The fragmentation pattern of lichenicidin wild type peptides (Figure 39) rather indicates that thioether rings are formed between Ser5 or Thr6 with Cys7 (Bliα) and Ser7 or Ser8 with Cys11 (Bliβ). MS/MS spectra of BliαS5A and BliαT6A, identified Bliα Ser5 as the amino acid involved in the Bliα A-ring formation. For Bliβ the bioactivity of BliβS7A suggests that Ser7 is the residue for Lan formation with Cys11. Recently, the structure of lichenicidin peptides Lchα and Lchβ isolated from *B. licheniformis* VK21 were elucidated by NMR (Shenkarev *et al.*, 2010). In general, the proposed structures are in agreement with the present work except for the A-ring of Bliα peptide. The MS/MS spectra obtained for Bliα of both *B. licheniformis* I89, *B. licheniformis* DSM13 and *E. coli* BLic5 exclude the occurrence of a

thioether ring between Thr3 and Cys7 due to the presence of an a_3-ion. Therefore, Bliα and Lchα may represent natural variants of lanthionine formation. Yet there is no experimental evidence that minor differences in dehydratase-cyclase could generate a structural variability, however, cluster alignment of LicM1 from *B. licheniformis* I89 (HQ290360) and *B. licheniformis* VK21 showed the presence of a 6 amino acid difference between both modifying enzymes.

Overall, 28 new lichenicidin variants were generated and expressed in *E. coli*, showing that the *trans* complementation system can be useful to generate other lichenicidin variants. As expected, bioactivity but also production was generally affected negatively when Lan or MeLan rings particularly located at the C-terminal were disturbed. These results are in accordance with those of lacticin 3147 *in vivo* SAR studies (Cotter *et al.*, 2006a). Moreover, the pattern of lanthionine rings (especially B, C and D rings) seems to be conserved among two-component lantibiotics, indicating their importance for the biological activity of these compounds.

This study showed that Gram-negative hosts are principally able to synthesize fully bioactive lantibiotics. Based on this system, amenable and time-saving procedures were developed to investigate the influence of *lic*-encoded proteins in the lichenicidin biosynthesis pathway and to produce new variants of lantibiotic peptides. Thereby, the present study initiates a new era in lantibiotics biosynthesis and bioengineering research employing Gram-negative hosts.

4.4 Experimental procedures

4.4.1 Bacterial strains, plasmids, general growth and procedures

Bacillus licheniformis I89 was grown at 37 °C with aeration in lichenicidin production medium M (see section 2.4.1, page55). *Escherichia coli* BW25113 and plasmids pIJ790 and pIJ733 were kindly supplied by Professor Mervyn Bibb (John Innes Centre, Norwich, UK). *E. coli* DH5α (Stratagene) was used as cloning host for plasmid constructions. Expression of lichenicidin variants was performed in *E. coli* BL21Gold® (Stratagene). The plasmid pET-24a(+) (Novagen) was the expression vector used for complementation studies and LanP expression and the plasmid pET15-b for the cloning of *tolC* gene. Fosmid pCC2FOS™ and *E. coli* EPI300™ (Epicentre) were the vector and host strain for the construction of *B. licheniformis* genomic library. *M.luteus* ATCC 9341 was the indicator strain for lichenicidin bioactivity. The properties of bacterial strains and plasmids used in this chapter are described in the Appendixes information. Also, the protocols for preparation of competent

cells, transformation, PCR purification, plasmid extraction and DNA purification from gels are presented in the same section.

Luria-Bertani (LB) medium was used for general liquid growth of *E. coli* strains, at 37 ºC or 30 ºC with aeration. Routine antibiotic selection was performed in LB agar plates containing the respective antibiotic concentrations.

4.4.2 Transformation of *B. licheniformis* I89

4.4.2.1 Transformation of *B. licheniformis* I89 protoplasts

The *B. licheniformis* I89 protoplasts transformation was carried out according with procedures described by (Cutting and Vander Horn, 1990; Waschkau *et al.*, 2008). The strain *B. licheniformis* I89 was inoculated in 25 mL of NBSG-X medium and grown overnight at 37 ºC, 200 rpm. The culture was diluted to an OD_{600nm} of 0.25 in 35 mL of the same medium and incubated in the same conditions until the OD_{600nm} reached 0.85-0.9. At this stage, the culture was transferred to a 50 mL sterile falcon and centrifuged at 4 ºC, 3220 x g for 15 min. The supernatant was discarded and the cells resuspended in 5 mL of SMMP solution supplemented with 0.30 mg/mL of lysozyme (prepared with SMMP; Roche). The mixture was incubated at 37 ºC at 90 rpm for 30 min and 12 mL of SMMP were added with gentle shaking. After centrifugation at 4 ºC, 420 x g for 12 min, the protoplasts were washed with 12 mL of SMMP solution and centrifuged again in the same conditions. The pellet was finally resuspended in 3 mL of SMMP solution and maintained on ice until the addition of plasmid DNA. The prepared protoplasts were transferred to a 1.5 mL sterile microcentrifuge tube containing a mixture of 25 µL of plasmid DNA (100 ng/µL) with 25 µL of 2x SMM solution. A 50 mL falcon was prepared with 1.6 mL of 40 % PEG 8000 (in 1x SMM) and the mixture of protoplasts-plasmid was transferred to it. After gently shaking at room temperature for 2 min, 5 mL of SMMP+ were added. The protoplasts were centrifuged at 8 ºC at 420 x g for 8 min, gently resuspended in 1 mL of SMMP+ solution and incubated for 2 hours at 30 ºC with aeration (100 rpm). Finally the same protoplasts were plated on three different agar plates, in duplicates. The agar plates included DM3 regeneration agar supplemented with chloramphenicol (Clo) at 25 µg/mL and without antibiotic and in LB agar. One duplicate was incubated at 30 ºC and the other at 37 ºC for 2 days.

Solutions:

Cryopreservation buffer: $CaCl_2$ 0.1 M, 15% (v/v) glycerol.

NBSG-X medium: Mix 500 mL of solution A with 500 mL of solution B. Shortly before use add 0.5 mL $FeCl_3.6H_2O$ (stock 8 mg/mL), 0.1 mL $MnSO_4.4H_2O$ (stock 2.5 mg/mL) and 2 mL of glycerol (stock 250 mg/mL).

Solution A: 56 g K_2HPO_4, 24 g KH_2PO_4, 2 g $(NH_4)_2SO_4$, pH 8 in a final volume of 500 mL.

Solution B: 3 g beef extract, 5 g bacto peptone, 1.2 g $MgSO_4.7H_2O$, 6 g trisodium citrate.$2H_2O$ in a final volume of 500 mL.

SMMP solution: Mix equal volume of 2x SMM and 4x PAB.

SMMP+ solution: 100 mL of SMMP with 0.2 mL of 20% (w/v) BSA (filter sterilized).

2x SMM: 1 M sucrose, 0.04 M sodium maleate, 0.04M $MgCl_2.6H_2O$.

0.2 N Sodium maleate: 5.8 g maleic acid , 50 mL of 1 N NaOH, in a final volume of 250 mL.

4x PAB: 6 g beef extract, 6 g yeast extract, 20 g peptone, 4 g dextrose, 14 g NaCl, 14.72 g K_2HPO_4, 5.28 g KH_2PO_4, in a final volume of 1 L.

DM3 regeneration agar: 0,8% (w/v) agar, 0.5 M sodium succinate [pH 7.3], 0.5% (w/v) casamino acids, 0.5% (w/v) yeast extract, 0.35% (w/v) K_2HPO_4, 0.15% (w/v) KH_2PO_4, 0.6% (w/v) glucose, 0.02 M $MgCl_2$. When the agar is at ~55ºC, 0.1% (w/v) BSA was added.

4.4.2.2 Transformation of *B. licheniformis* I89 electrocompetent cells

The electroporation of *B. licheniformis* I89 cells protocol was constructed by the adaptation of procedures described by (Brigidi *et al.*, 1991). The strain *B. licheniformis* I89 was inoculated in 20 mL of and grown overnight at 37ºC with aeration (160 rpm). 10 mL of this culture was inoculated in 100 mL of SPII medium and grown until an OD_{600nm} of 0.6. At this stage, the culture was centrifuged at 4000 x g at 4 ºC for 5 min and washed with 100 mL of sterile ice-cold ultrapure water. This step was repeated three times and the final wash step was performed with 20 mL of ice-cold 30 % (w/v) PEG 4000 and the cells were centrifuged in the same conditions described above. Finally, the cells were resuspended in 200 μL of the same solution and kept on ice until the addition of plasmid DNA. The plasmid DNA (5 μL of 1 mg/mL) was added to 40 μL of the prepared cells on ice. The cell-plasmid mixture was transferred to an ice-cold electroporation cuvette with an electrode gap of 0.2 cm. The mixture was pulsed in the Bio-Rad GenePulser II® equipment with the following parameters: 2500 V, 25 μF and the parallel resistor at 400 Ohms. After the pulse, the cells were immediately diluted in 1 mL of ice-cold SOC broth and incubated at 30 ºC for 2 hours with aeration (160 rpm). Finally, the cells were centrifuged at 2500 x g for 1 min, resuspended in 100 μL of the same medium and plated on nutrient agar (Difco) supplemented with 1 % (w/v) sodium pyruvate and chloramphenicol (25 μg/mL).

Solutions:

SPII: 14 g K_2HPO_4, 6 g KH_2PO_4, 0.2 g $MgSO_4$ in 1L final volume. This medium was prepared as 2X solution and the following components were added before bringing the medium up to its final volume: 10 mL of 10% (w/v) glucose, 2.5 mL of 1M $MgCl_2$, 1 m3 of o.5 M $CaCl_2$.

SOC: 2% (w/v) tryptone; 0.5% (w/v) yeast extract, 10 mM NaCl; 2.5 mM KCl; 10 mM $MgCl_2$, 10 mM $MgSO_4$.

4.4.3 Construction and screnning of I89 strain fosmid library

The construction of a fosmid library implicated the extraction of high molecular weight DNA from the *B. licheniformis* I89 strain. The screening of the library was performed by colony-blot hybridization using colony blot hybridization with a DIG DNA labeled probe.

4.4.3.1 Extraction of high molecular weight DNA

The extraction of high molecular weight DNA was performed as follows: an overnight culture was diluted 1/100 in 200 mL of medium M supplemented with 1 % glycerol and incubated at 37 °C with aeration until OD_{600} of 0.5. The cells were harvested at 4,000 x g for 5 min, resuspended in 5 mL of TES solution and incubated at 37 °C for 2 hours. The lysate was slowly added to a tube containing 10 mL of EPS solution, followed by gentle mixing and incubated at 50 °C for 1 hour. Deproteinization was performed by phenol/CIA (Invitrogen) extraction followed by centrifugation of 15 min at 2,000 x g. DNA precipitation was performed by an adaptation of the protocol described by Akamatsu *et al.* (2000). Briefly, 1/10 volumes of a 3 M sodium acetate solution (pH 5.2) were added to the aqueous phase obtained from the phenol/CIA extraction and the solution chilled on an ice bath. Subsequently, 2 volumes of cold ethanol (- 50 °C) were added to the solution. The visible precipitated DNA was recovered from the mixture with a glass pipette, washed with 70 % ethanol and finally resuspended in 100 μL of sterile distilled water containing 1 mg/mL of RNase. This DNA was used for the construction of the *B. licheniformis* I89 fosmid library using the CopyControl™ Fosmid Library Production Kit (Epicentre) according with the manufacturer's instructions (Appendix 9).

Solutions:

TES: 20 mM Tris/HCl, pH8.0; 25 mM EDTA; 10% saccharose

EPS: 100 mM EDTA, pH8.0; 1% sarcosyl; 50 μg/mL proteinase K

4.4.3.2 Colony-blot hybridization

The screening for the presence of *licA1* gene in the clones constituting the *B. licheniformis* I89 fosmid library was performed by colony-blot hybridization using a DIG DNA labeled probe as an adaptation of the protocol previously described by Ferreira (2010) . Briefly, the nitrocelluse membranes (Amersham) with 90 mm of diameter were soaked for 5 min with lysis solution and placed on the plates containing the clones. The membranes were previously marked with a razor to allow the identification of the clone

numbers in the final of the procedure. The membranes were lifted off carefully and placed side up. Two Whatmann 3MM filters were separately soaked with lysis solution and denaturing solution. The membranes were placed side up in the lysis solution filter for 5 min and subsequently in the denaturing solution filter for another 5 min. The excess of the respective solutions was always removed with a clean Whatmann 3MM filter. Then, the membranes were floated side up on neutralizing solution for 5 min in a petri dish. After this period the membranes were submerged in this solution and the cellular debris was carefully washed with the fingers. The excess of solution was removed and the membranes submerged again in a 6X SSC solution for 5 min present in other petri dish. The excess of solution was removed and the DNA fixed for 45 sec under UV light. The membranes were placed into a hybridization tube and treated with 10 mL of pre-hybridization solution, avoiding foam formation, at 40 ºC for 3 hours. The labelled DNA probe (see section 4.4.3.3 below) was denatured by boiling for 10 min and promptly cooled on ice. This probe was added to the tube and the membranes incubated overnight at 65 ºC. The membranes were washed twice for 5 min with washing solution I at room temperature and twice for 15 min with washing solution II at 68 ºC, always with agitation. After what, the membranes were incubated with acid maleic buffer for 5 min, with 100 mL of 1X blocking solution for 30 min and another 30 min in 200 mL of antibody solution, always at room temperature. The membranes were washed twice for 15 min with another 100 mL of maleic acid buffer and equilibrated for 5 min with 20 mL of detection buffer. Finally, the membranes were placed in a plain dish and for detection, were incubated in 10 mL of freshly prepared colour substrate solution, in the dark. The reaction was stopped washing the membrane for 5 min with 50 mL of distilled water.

Solutions:

Lysis solution: 5% (w/v) SDS.

Denaturing solution: 1.5M NaCl in 0.5M NaOH.

Neutralizing solution: 0.5 M Tris-HCl, 1.5 M NaCl, pH 7.6.

20X SSC solution: NaCl 3 M, sodium citrate 0.3 M, pH 7.0.

Pre-hybridization solution: 40% (v/v) formamide, 5% SSC, 2% (w/v) blocking reagent (Roche), 0.02% (w/v) SDS, 0.1% (w/v) N-lauroylsarcosine.

Solution I: 2X SSC, 0.1% (w/v) SDS.

Solution II: 0.5X SSC, 0.1% (w/v) SDS.

Acid maleic buffer: 0.1 M maleic acid, 0.15 M NaCl, pH 7.5.

1X Blocking solution: dilution of 10X blocking solution (Roche).

Antibody solution: dilution of anti-digoxigenin-AP 1:5000 in 1X blocking solution (Roche).

Detection buffer: 0.1 M Tris-HCl, 0.1 M NaCl, 50 mM MgCl2, pH 9.5.

Colour substrate solution: 40 μL of NBT/BCIP solution (Roche) in 2 mL of detection buffer.

4.4.3.3 DNA probe labelling

The DNA probe used for the screening of the fosmid library consisted of the DIG labeled *licA1* gene. The *licA1* gene used for the labeling was obtained by PCR in a final volume of 25 μl containing 1.5 mM of $MgCl_2$, 200 μM of dNTP's, 1X Green GoTaq® Buffer, 0.25 U of GoTaq® Buffer (Promega) and 0.3 pmol/μL of each primer. 1 μL of I89 bacterial suspension was applied as DNA template. The amplification parameters were as follows: 94 ºC for 5 min, thirty-five cycles of 94 ºC for 30 sec, 50 ºC for 30 sec and 72 ºC for 30 sec and a final extension step of 10 min at 72 ºC. The primers used for the amplification were mrsAfw (5'-AAAAAGGAAATGATTCTTTCATGG-3') and mrsArv (5'-AGCTTGGCATGCATTCTTTT-3'). The presence of the correct amplicon (approximately 220 bp) was evaluated on 1 % agarose gel electrophoresis. Approximately 1 μg of amplified DNA was diluted in distilled water for a final volume of 16 μL and denatured by boiling for 10 min in a water bath and quickly chilled on ice. 4 μL of Dig-High Prime solution (Roche) was added to the DNA. The mixture was incubated at 37ºC overnight. The reaction was stopped heating to 65ºC for 10 min. The labeled probe was stored at -20ºC until its addition to the membranes (see section 4.4.3.2).

4.4.4 Lichenicidin heterologous expression in *E. coli*

E. coli clones which were positive for the presence of *licA1* structural gene were screened for lichenicidin production by colony bioassay as described in 2.4.8 (page number 61). All the clones present antibacterial activity, indicating the presence of all the lichenicidin biosynthetic gene cluster in the corresponding fosmids. In order to select the best clone for further studies, the stability of production without selective pressure was assessed through sequential subcultures of the *E. coli* clones on bioassay agar plates. Based on these assays and sequencing, clone *E. coli* Lic5 possessing the fosmid p*Lic5* was selected.

The evaluation of lichenicidin production by *E. coli* Lic5 was performed in medium M. A pre-culture was performed at 37 ºC along 16 hours with aeration (160 rpm). 500 μL of this culture were used to inoculate 50 mL of fresh medium and allowed to grow in the same conditions for 36 hours. Along this period, 1 mL samples were withdrawn from this culture and centrifuged for 5 min at 16100 x g. The cell-free supernatant was stored at -20 ºC while cells were resuspended in 100 μl of 70 % isopropanol containing 0.1 % of formic acid. After 2 hours at 4 ºC, the mixture was centrifuged for 5 min at 16100 x g, the respective supernatant collected for a new microtube and stored at -20 ºC. The antibacterial activity of the supernatant and the cell wall washes was evaluated through the application of

40 μl of each fraction to bioassay plates as described on 2.4.8 of page 61. Samples exhibiting the major inhibition zone were selected for LC-ESI-MS analysis. The antibacterial activity and LC-ESI-MS analysis of negative controls consisting of *E. coli* EPI300 strain harboring the empty pCC2FOS vector were also performed.

4.4.5 Extraction of fosmid DNA from *E. coli*

The extraction of fosmid DNA was performed accordingly with an adaptation of the alkaline lysis procedure described by Sambrook and Russell (2001). Briefly, a 50 mL of LB containing 12.5 μg/mL of chloramphenicol were inoculated with the desired strain and cultured overnight at 37°C, with aeration (160 rpm). 40 mL of this culture was centrifuged at 12000 x g for 1 min and the cells were resuspended in 1 mL of Solution I, containing 20 μg/mL of DNase-free RNase A (Roche). To perform the cell lysis, 1.6 mL of freshly prepared Solution II was added and the tube shacked vigorously 5 times. 1.2 mL of the alkaline Solution III was subsequently added; the solution was gently mixed and incubated on ice for 5 min. The separation of the cell debris and chromosomal DNA was performed by centrifugation at top speed for 5 min. The supernatant containing the fosmid DNA was extracted twice with Phenol/CIA (Invitrogen). The DNA was precipitated adding 1/10 volumes of solution III and 0.6 volumes of isopropanol. The mixture was incubated at room temperature for 30 min and centrifuged at top speed for 15 min at 4 °C. The pellet was washed with 500 μL of 70 % of ethanol and a last step of centrifugation at top speed for 5 min was performed. The precipitate DNA was dried in the flow chamber for 15 min to evaporate the residual ethanol and resuspended in 100 μL of distilled water. Alternatively, it is also possible to extract efficiently fosmid DNA for transformation from 10 mL of initial culture, using the solutions of GeneJET™ Plasmid Miniprep Kit but without the application of the columns, performing the DNA precipitation with isopropanol.

4.4.6 Lichenicidin gene cluster knockout mutants

In frame gene deletions of lichenicidin biosynthetic cluster were performed using the λ-Red-mediated recombination system adapted from Gust *et al.* (2003).

4.4.6.1 Digestion of pIJ733 plasmid

The disruption cassette chosen to inactivate genes constituting the *lic* gene cluster was amplified from the pIJ733 plasmid (Appendix 3) and was consisted by an apramycin (Apra) resistance gene. In order to decrease the occurrence of false positives, the pIJ733

plasmid was digested before its use as template for the PCR reaction. Thus, the plasmid was extracted from the *E. coli* BW25113/pIJ773 with the GenJet plasmid DNA purification kit (Fermentas), accordingly with manufacturer's instructions (Appendix 6). Approximately 10 μg of the resulting plasmid was digested with 50 U of *Eco*RI and 50 U of *Hind*III restriction enzymes (Fermentas) in a 100 μL total volume reaction containing 1X of buffer R (Fermentas). The digestion was loaded into a 1 % agarose gel and run at 90 V during 2 hours. The band containing the apramycin cassette (approximately 1400 bp) was purified from the agarose gel with the QIAquick gel extraction kit (Quiagen), accordingly with the manufacturer's instructions (Appendix 7). The cassette was stored at -20 ºC until further use as DNA template for amplification reaction.

4.4.6.2 Design of long PCR primers and amplification of the disruption cassette

For the amplification of the Apra cassette applied for each *lic* gene disruption it was necessary to construct of two long PCR primers (58 and 59 bp). Each one of these primers possessed at the *5´*-end, 39 bp matching the sequence adjacent to the gene to be inactivated, and the 3´-sequence (19 nt or 20 nt) matching the 5'- (for forward primer) or the 3'- (for reverse primer) region of the Apra resistance cassette. All the constructed primers were compared with the p*Lic5* fosmid, where a perfect match was identified in the flanking regions of the gene to be interrupted. If other matches are identified >30 bp, a new primer was constructed. The primers used in this study were designed in order to excise the entire gene, letting stand only its start and stop codon. In some cases, this criterion could not be applied do to the presence of putative RBS or promoters of other genes. In this situation, primers were designed to in order to excise the maximum of the gene possible. A *Bmt*I restriction site (GCTAG↓C) was also included in both primers between the sequence of the disruption cassette and the sequence of the flanking region of the target *lic* gene to allow posterior removal of the Apra resistance cassette.

The primers used for amplification of each Apra resistance cassette are listed on Table 8. Each amplification reaction was performed in a final volume of 50 μL containing 1X of Herculase II reaction buffer, 0.25 mM of dNTP's, 3 pmol/μL of each primer and 0.25 μL of Herculase® II fusion DNA polymerase (Agilent Technologies). Approximately 100 ng of the purified Apra^R cassette obtained in the previous section was used as DNA template for each reaction. The amplification parameters were as follows: one step of denaturation at 94 ºC for 45 sec followed by 10 cycles of denaturation at 94 ºC for 45 sec, primer annealing at 50 ºC for 45 sec and extension at 72 ºC for 90 sec. This was followed by 15 cycles of denaturation at 94 ºC for 45 sec, primer annealing at 55 ºC for 45 sec and extension at

72 °C for 90 sec. Finally a final extension step at 72 °C for 5 min was included. Two reactions involving the same primers were purified and concentrated into a 20 μL final volume using the GenJet PCR purification kit. The amplification products (approximately 1400 bp) were always analyzed by gel electrophoresis and stored at -20 °C until further use.

Table 8: List of primers used to perform the interruption of biosynthetic genes involved in the production of lichenicidin peptides. The restriction site for *Bmt*I digestion was highlighted in bold to better distinguish between the sequence of the disruption cassette for that of the flanking regions of the target *lic* gene. These primers were used in the procedure described in section 4.4.6.2.

Target gene	Primers	
	Name	Sequence (5'→3')
licM1	lanM1_fw	CGCGTTGGCATATTGATAGAAAAAGAGGTCGATAGAATG**GCTAGC**ATTCCGGGGATCCGTCGACC
	lanM1_rv	AAAAAATCTATGGATGAAAATCCATAGATTTTGATTTTA**GCTAGC**TGTAGGCTGGAGCTGCTTC
licA1	lanA1_fw	GACAAAAAATTATAAATTTTCTAGGAGGTGGAATATATG**GCTAGC**ATTCCGGGGATCCGTCGACC
	lanA1_rv	ATGCCAACGCGGGAGCAGGGCCCCCGCGTTGGGAACTTA**GCTAGC**TGTAGGCTGGAGCTGCTTC
licA2	lanA2_fw	TGCAAGGATGGATTCTTTTGAATTTTTTATGATTCCCTA**GCTAGC**ATTCCGGGGATCCGTCGACC
	lanA2_rv	ATTTCGATAGTTTGCCCGTTCTAGGAGGTGAGAATCATG**GCTAGC**TGTAGGCTGGAGCTGCTTC
licM2	lanM2_fw	CTATAAACGGTGTCTTATGAAAAAACAAGCCTTATCTCA**GCTAGC**ATTCCGGGGATCCGTCGACC
	lanM2_rv	GCAGGGATGGTTTTCTTCGCCAAAGGGATGACGAGAATG**GCTAGC**TGTAGGCTGGAGCTGCTTC
licT	lanT_fw	TTTTTTCATAAGACACCGTTTATAGAACAGATGCAGCAG**GCTAGC**ATTCCGGGGATCCGTCGACC
	lanT_rv	TTGAATGTCATCGTATATTTGCGCCATTTTTGCGACTTC**GCTAGC**TGTAGGCTGGAGCTGCTTC
licP	lanP_fw	GGCGGAAAGACGGCTCAAGCAAAAGAACAAGCAGGAGAA**GCTAGC**ATTCCGGGGATCCGTCGACC
	lanP_rv	AGCTACCTGCGGGGCGGCCAAGCTTGTTCCGTATGAGAG**GCTAGC**TGTAGGCTGGAGCTGCTTC
licX	lanX_fw	TCAAGCGCTGAAAATGATGAACAAGGAGTGAAGGGTATG**GCTAGC**TGTAGGCTGGAGCTGCTTC
	lanX_rv	CTTCACGTGATGATTGTGTGAAGATCTTTTCGACAATCA**GCTAGC**ATTCCGGGGATCCGTCGACC
licR	lanR_fw	TTTTTGTTATAAACTCTTTACAATGTGTAAAAAACATTG**GCTAGC**TGTAGGCTGGAGCTGCTTC
	lanR_rv	TCCTTCTCAAATAACGCGGCAATGCGAAACCCCATTAAC**GCTAGC**ATTCCGGGGATCCGTCGACC
licY	lanY_fw	GGTATTTCAATACCAACTAGAGGAGGATGATCGCTGATG**GCTAGC**TGTAGGCTGGAGCTGCTTC
	lanY_rv	TGTGACCATGTTTTTATAATTGGCGAACGACAAATCGAC**GCTAGC**ATTCCGGGGATCCGTCGACC

4.4.6.3 Disruption of *lic* genes

The disruption of lichenicidin biosynthetic genes was performed in the fosmid pLic5. This plasmid was transformed in electrocompetent *E. coli* BW25113/pKD20 cells resistant to ampicillin (Amp) due to the plasmid pKD20, which replication is sensitive to temperatures superior to 32 °C. This strain is amenable to the introduction of foreign linear DNA. The resulting strain *E. coli* BW25113/pKD20/pLic5 was characterized by Amp^R and Clo^R and were always grown at 30 °C. The introduction of each amplified disruption cassette in the

E. coli BW25113/pKD20/pLic5 strain was performed as follows: a pre-culture of this strain was performed in LB medium containing 100 μg/mL of Amp and 12.5 μg/mL of Clo antibiotics. 10 mL of fresh LB medium containing the same concentration of the selective markers, 20 mM of $MgSO_4$ and 10 mM of L-arabinose (Sigma) were inoculated with 100 μL of the previously prepared culture. The cells were grown at 30 °C at 160 rpm until an OD_{600} of approximately 0.4 (between 3 to 4 hours). The cells were collected by centrifugation at 6000 x g for 5 min at 4 °C and washed with 10 mL of ice cold 10 % glycerol. This procedure was repeated once and the cells finally resuspended on 100 μL of the same solution.

Table 9: Sequence of primers used for the confirmation of the substitution of the *lic* genes by the apramycin resistance cassette and PCR parameters for the corresponding amplifications. The same primers were used for the confirmation of the resistance cassette deletion.

Target gene	Primers		PCR parameters	
	Designation	Sequence (5'→3')	Annealing temperature	Extension time
licM1	check_licM1_fw	CTAGAACGGGCAAACTATCG	52 °C	3 min 30 sec
licA1	check_licM1_rv	ACGGCGGATACTGAATGGTG		
licA2	check_licM2_fw	ATACAGCACAGTCCGCATTC		
licM2	check_licM2_rv	GTTCCCTGCTGGATGATAAG		
licT	check_licT_fw	TGCTCCTACAGCGATTGAAA		
licP	check_licT_rv	AATAGCCTACGGGCTTTTGC		
licX	check_Reg_fw	TTTCCCGATCCGTTTTCTCC	54 °C	45 sec
licR	check_Reg_rv	CCTGAGGAAATCGGCATTAG		
licY				

Table 10: Molecular size of the amplifications expected for the screnning of the substitution of the Apra resistance cassette for each *lic* gene and for its posterior removal.

Target gene	Amplification expected		
	Gene intact	Apra substitution	Apra removal
licM1	3800 bp	2100 bp	640 bp
licA1		5030 bp	3570 bp
licA2	3668 bp	2011 bp	551 bp
licM2		4800 bp	3420 bp
licT	3616 bp	4055 bp	2595 bp
licP		3370 bp	1914 bp
licX	1108 bp	2320 bp	920 bp
licR		2230 bp	830 bp
licY		2221 bp	821 bp

For transformation, 50 μL of the prepared cells were mixed with 100-150 ng of the Apra disruption cassette obtained in section 4.4.6.2. The cells were subject to electroporation, where the one hour expression step was performed at 30 ºC and the transformants finally selected on LB agar plates containing Apra at 50 μg/mL and Clo at 12.5 μg/mL grown at 37 ºC (since no other interruptions were intended). The substitution of the target lic gene by the Apra^R cassette was confirmed by colony PCR using the primers described on Table 9. The amplification was performed as follows: 94 ºC for 5 min, 35 cycles of 94 ºC for 30 sec, amplification temperature for 30 sec and 72 ºC for extension time and a final extension step of 10 min at 72 ºC, where the amplification temperature and extension times applied were described on Table 9. The size of the expected amplifications is presented on Table 10. The DNA template used corresponded to one colony of each transformant to be screened. In all the reactions, the unchanged p*Lic5*fosmid was used as control.

4.4.6.4 Elimination of Apra disruption cassette

The disrupted fosmids produced in the previous section were extracted by alkaline lysis as described in section 4.4.5 and digested with the restriction enzyme *Bmt*I (New England Biolabs). The reaction was performed in a final volume of 80 μL, containing 1-3 μg of fosmid DNA, 1X of NEBuffer 2 and 20 U of enzyme. The mixture was incubated at 37 ºC for 3 hours. Subsequently, sterile distilled water as added to the digestion for a final volume of 600 μL. This mixture was extracted once with Phenol/CIA (Invitrogen) and 1/10 vol of potassium acetate (3 M, pH 5.5) and 0.6 vol of isopropanol were added to the aqueous DNA-containing phase. The mixture was incubated at room temperature for 15 min and centrifuged at 4 ºC, 12000 x g during 15 min. The pelleted DNA was washed with 100 μL of 70 % ethanol and completely dried for 15 min in the flow chamber. The final elution was performed in 10 μL of sterile distilled water. The complete digestion of the fosmid was confirmed by gel electrophoresis analysis, loading 1 μL of the digested DNA. If the fosmid was not completely digested, a new reaction was performed as described above. The religation of the *Bmt*I-digested fosmid was performed in a total volume of 50 μL containing approximately 1-2 μg of DNA, 1X ligase buffer and 10 U of T4 DNA ligase (Fermentas). The reaction was incubated at 20 ºC for 15 min and 5 μL of this ligation was use to transform chemically competent *E. coli* EPI300 cells. The transformants were selected on LB agar plates containing Clo (12.5 μg/mL). The obtained colonies were further cultured on plates containing Clo (12.5 μg/mL) and Apra (50 μg/mL). The clones presenting the phenotype Clo^{R}Apra^S were selected as those containing the desired gene deletion without the Apra^R

cassette. The absence of this cassette was further confirmed by colony-PCR as described in section 4.4.6.3. Finally, the PCR product was submitted to sequencing reaction in order to confirm the deletion of each gene.

The strains resulting from this procedure were *E. coli* Lic5Δ*A1*, Lic5Δ*A2*, Lic5Δ*M1*, Lic5Δ*M2*, Lic5Δ*T*, Lic5Δ*P*, Lic5Δ*X*, Lic5Δ*R* and Lic5Δ*Y* knockout mutants where the *licA1*, *licA2*, *licM1*, *licM2*, *licT*, *licP*, *licX* and *licY* genes were deleted from p*Lic5*, respectively.

4.4.6.5 Preparation of extracts for LC-ESI-MS analysis and bioassays

A pre-culture of each knockout strain was performed in medium M supplemented with the appropriate selective agent. 500 μL of this culture were added to 50 mL of fresh medium M without addition of antibiotics and allowed to grow at 37 ºC for 24 hours with aeration (160 rpm). The supernatant was collected by centrifugation at 10,000 x g for 5 min, filter-sterilized and stored at 4 ºC. The cells were collected by centrifugation at 10,000 x g for 5 min and resuspended with 70 % of isopropanol containing 0.1 % of formic acid. The solution was stored at 4 ºC for 1 hour and subsequently centrifuged at 10,000 x g for 5 min. The obtained supernatant corresponded to the cell wall wash fraction. To the cell-free culture supernatant (40 mL), 2 mL of 1-butanol (Merck) was added and the solution stirred for 3 hours at room temperature. Then the mixture was centrifuged for 1 min at 3,000 x g and the upper organic phase collect to a new tube, constituting the butanol extract. In order to investigate the production of lichenicidin peptides by the knockout strains, both extracts from each strain were analyzed by LC-ESI-MS.

All the lichenicidin knockout strains were tested for their antagonistic ability by colony-bioassay as described in section 2.4.8 of page 61. Complementation of bioactivity was also tested, where knockout mutants' colonies were inoculated next to *E. coli*Lic5Δ*A1*and *E. coli* Lic5Δ*A2* colonies on bioassay agar plates (section 2.4.8 of page 61). *E. coli* Lic5Δ*A1*and *E. coli* Lic5Δ*A2* represented the exclusive production of Bliβ and Bliα, respectively.

4.4.7 LicP proteolysis analysis

In order to investigate the potential extracellular location and involvement of LanP enzyme in Bliβ proteolysis, a simple experiment involving the single expression of *lanP* in *E. coli* and also the exposition of *E. coli* Lic5Δ*P* cell wall washes and supernatant extracts to LanP was performed.

4.4.7.1 Construction of BL21*licP*$^+$ strain

The *lanP* gene (approximately 1300 bp) was amplified with comp_lanPfw (5'-TATACA**TATGAA**AAGAATATATATTT-3') and comp_lanPrv (5'-TTTTAT**CTCGAG**TCACTCCTTGTTCATCATTTT-3') primers, which contained the *Nde*I and *Xho*I restriction enzymes recognition sequence (bold), respectively. Each amplification reaction was performed in a final volume of 50 μL containing 1X of Herculase II reaction buffer, 0.25 mM of dNTP's, 3 pmol/μL of each primer and 0.25 μL of Herculase® II fusion DNA polymerase (Agilent Technologies). The amplification parameters were as follows: 94 ºC for 5 min, thirty-five cycles of 94 ºC for 30 sec, 48 ºC for 30 sec and 72 ºC for 1 min 30 sec and a final extension step of 10 min at 72 ºC. 1 μL of a *B. licheniformis I89* suspension was used as DNA template. Two of such reactions were performed, pooled together and concentrated by final elution with 30 μL of sterile distilled water with the GenJet PCR purification kit (Fermentas). The PCR product was further digested with 10 U of *Nde*I and 20 U of *Xho*I (Fermentas). The reaction contained approximately 1-2 μg of purified PCR product and 1X of Buffer O (Fermentas), in a final volume of 40 μL. The pET24-a(+) vector was also digested in the same conditions. The reactions were incubated at 37 ºC for 3 hours, and purified with the GenJet PCR purification kit (Fermentas). Approximately 50 ng of the linear vector was ligated to 150 ng of digested PCR-product in a 20 μL reaction containing approximately 1-2 μg of DNA, 1X ligase buffer and 10 U of T4 DNA ligase (Fermentas). 5 μL of this reaction was used to transform chemically competent *E. coli* BL21Gold cells and the correspondent transformants selected on LB agar plates containing kanamycin (Kan) at 50 μg/mL. Positive clones were screened by colony-PCR using the T7prom (5'-TAATACGACTCACTATAGGGAGACCAC-3') and T7term (5'-CAAAAAACCCCTCAAGACCC-3') primers. Each PCR reaction was performed on a final volume of 12.5 μL containing containing 1.5 mM of $MgCl_2$, 200 μM of dNTP's, 1X Green GoTaq® Buffer, 0.25 U of GoTaq® Buffer (Promega) and 0.3 pmol/μL of each primer. The amplification parameters were as follows: 94 ºC for 5 min, 35 cycles of 94 ºC for 30 sec, 54 ºC for 30 sec and 72 ºC for 1 min 30 sec and a final extension of 10 min at 72 ºC. The resulting p*licP* plasmid was extracted from its harboring BL21*licP*$^+$ strain and sequenced to confirm the absence of mutations. The empty pET24-a(+) vector was also transformed into chemically competent *E. coli* BL21Gold cells to obtain the control strain BL21*licP*$^-$.

4.4.7.2 Preparation of LicP-containing extracts and bioassay

Both BL21*licP*$^+$ and BL21*licP*$^-$ strains were separately cultivated in 50 mL of medium M without antibiotic selection, at 37 ºC during 24 hours with aeration (160 rpm). The supernatant was collected by centrifugation at 10,000 x g for 5 min, filter-sterilized and

stored at 4 ºC. The *E. coli* Lic5Δ*P* knockout mutant was cultured and its extracts obtained as described in section 4.4.6.5. The *E. coli* Lic5Δ*P* cell wall washes and butanol extracts were bioassayed side by side with the *E. coli* BL21*licP*$^{+}$ and *E. coli* BL21*licP*$^{-}$ supernatants on plates containing the indicator strain.

4.4.8 *trans* complementation system

In order to produce lichenicidin peptides variants, a *trans* complementation system in *E. coli* BL21Gold® for the Lic5Δ*A1* and Lic5Δ*A2* knockout mutants was established.

To archive this, the fosmids pLic5Δ*A1* and pLic5Δ*A2* fosmids were extracted from *E. coli* Lic5Δ*A1* and *E. coli* Lic5Δ*A2* strains respectively as described in section 4.4.5. Subsequently they were transformed into chemically competent *E. coli* BL21Gold cells and positive clones were selected on LB agar plates containing Clo at 12.5 μg/mL. The resultant strains were designated as *E. coli* BLic5Δ*A1* and BLic5Δ*A2*, which were subsequently transformed with the plasmids p*licA1* and p*licA2*, respectively.

The p*licA1* and p*licA2* plasmids were obtained by amplification of *licA1* and *licA2* with the primers listed on Table 11. Each PCR reaction was performed in a final volume of 50 μL containing 1X of Herculase II reaction buffer, 0.25 mM of dNTP's, 3 pmol/μL of each primer and 0.25 μL of Herculase® II fusion DNA polymerase (Agilent Technologies). The amplification parameters were as follows: 94 ºC for 5 min, 35 cycles of 94 ºC for 30 sec, amplification temperature for 30 sec and 72 ºC for 45 sec and a final extension step of 10 min at 72 ºC. The amplification temperature applied is listed on Table 11. 1 μL of a *B. licheniformis I89* suspension was used as DNA template. Two of each of such reactions were performed, pooled together and concentrated by final elution with 30 μL of sterile distilled water with the GenJet PCR purification kit (Fermentas). Each PCR product was further digested with 10 U of *Bam*HI and 20 U of *Xho*I (Fermentas). The reaction contained approximately 1-2 μg of purified PCR product and 1X of Buffer *Bam*HI (Fermentas), in a final volume of 40 μL. The pET24-a(+) vector was also digested in the same conditions. The reactions were incubated at 37 ºC for 3 hours and purified with the GenJet PCR purification kit. 50 ng of linear vector was ligated to 150 ng of digested PCR-product in a 20 μL reaction containing 1X ligase buffer and 10 U of T4 DNA ligase (Fermentas). 5 μL of this reaction was used to transform chemically competent *E. coli* DH5α cells and the correspondent transformants selected on LB agar plates containing Kan at 50 μg/mL. Positive clones were screened by colony-PCR using the T7prom (5'-TAATACGACTCACTATAGGGAGACCAC-3') and T7term (5'-CAAAAAACCCCTCAAGACCC-3') primers. Each PCR reaction was performed on a final

volume of 12.5 μL containing containing 1.5 mM $MgCl_2$, 200 μM dNTP's, 1X Green GoTaq® Buffer, 0.25 U of GoTaq® Buffer (Promega) and 0.3 pmol/μL of each primer. The amplification parameters were as follows: 94 ºC for 5 min, thirty-five cycles of 94 ºC for 30 sec, 54 ºC for 30 sec and 72 ºC for 45 sec and a final extension of 10 min at 72 ºC. One positive clone for each construction was selected and the p*licA1* and p*licA2* plasmids extracted with the GenJet plasmid purification kit (Fermentas). Both plasmids were sequenced in order to confirm the absence of mutations.

Approximately 50 ng of the plasmids p*licA1* and p*licA2* were transformed into chemically competent *E. coli* BLic5Δ*A1* and BLic5Δ*A2* strains. Positive clones were selected on LB agar plates supplemented with 12.5 μg/mL of Clo and 50 μg/mL of Kan with incubation at 37 ºC. From each transformation plate five clones were randomly selected and tested for presence of antibacterial activity by colony bioassay as described in section 2.4.8 (page 61). One representative of each transformation possessing restored antibacterial activity was selected for further applications and designated as BLic5Δ*A1*p*licA1* and BLic5Δ*A2*p*licA2*. The production of both lichenicidin peptides by BLic5Δ*A1*p*licA1* and BLic5Δ*A2*p*licA2* strains was also evaluated by LC-ESI-MS analysis. The extracts used for this analysis were prepared as described in section 4.4.6.5.

Table 11: List of primers and respective amplification temperatures used for amplify *licA1* and *licA2* genes to clone into the vector pET24-a(+). The expected amplification was also included.

Target	Designation	Primer Sequence (5' → 3')	Amplification temperature	Expected size of amplification
licA1	Comp_licA1fw	AGGTGGGATCCATGTCAAAAAAGGAAATG	50 ºC	250 bp
	Comp_licA1rv	CCCGCCTCGAGAACTTAGTTACAGCTTGGC		
licA2	Comp_licA2fw	ATCAGGATCCATGAAAACAATGAAAAATTCAG	59 ºC	239 bp
	Comp_licA2rv	TTTTATCTCGAGCTAGCATCGGCTTGTACAC		

4.4.9 Production of lichenicidin variants

Alanine scanning of Bliα and Bliβ peptides was performed by site-directed mutagenesis of *licA1* and *licA2* genes, using a modification of the two-step reactions method described by Wang *et al.* (1999), using p*licA1* and p*licA2* as templates. Primers used to insert the desired mutation were designed with web-based software PrimerX (http://www.bioinformatics.org/primerx/) and are listed on Table 12 and Table 13.

The site-directed mutagenesis PCR was performed in two distinct reactions, each one containing exclusively the forward or the reverse primer. Each reaction was performed in a final volume of 25 μL containing 1X of Herculase II reaction buffer, 0.25 mM of dNTP's,

3 pmol/μL of the primer, 50 ng of p*licA1* or p*licA2* DNA and 0.25 μL of Herculase® II fusion DNA polymerase (Agilent Technologies). The of amplification was performed by a initial denaturation step of 95 ºC for 3 min, followed by 5 cycles of denaturation at 95 ºC for 20 sec, primer annealing at 55 ºC for 30 sec and amplification at 72 ºC for 3 min and a final amplification step of 3 min. At the end, both reactions were mixed together in the same tube and a second stage of amplification was executed with the same parameters described for the first stage, however including 20 cycles of amplification. Subsequently, each PCR reaction was digested with 10 U of *Dpn*I (Fermentas) and incubated at 37 ºC for 1 hour, in order to digest the parental methylated plasmid DNA. Finally, 5 μL of this reaction was used to transform chemically competent *E. coli* DH5α cells and transformants selected overnight on LB agar plates containing 50 μg/mL of Kan. For each reaction, 3 Kan^R clones were selected and plasmid DNA extracted with the GenJet plasmid DNA Miniprep kit (Fermentas). Each plasmid was sequenced using the primers T7prom and T7term to confirm the presence of the desired mutation. The mutated plasmid was used to transform chemically competent BLic5*ΔA1*or BLic5*ΔA2* cells, depending if permutation of amino acid was done for *licA1* or *licA2* genes, respectively. The transformants were selected on LB agar plates containing 12.5 μg/mL of Clo and 50 μg/mL of Kan antibiotics.

Table 12: List of primers designed on PrimerX web-based software used to introduce the desired mutation on *licA1* peptide, where the first letter represents the aminoacid to be substituted by an alanine residue.

Mutation	Primers (5' → 3')
T1A	A1T1Afw: CATCGCCGGAGGCGCAATCACGCTCAG A1T1Arv: GTAGCGGCCTCCGCGTTAGTGCGAGTC
T3A	A1T3Afw: CATCGCCGGAGGCACAATCGCACTCAGCACTTGTGCCATC A1T3Arv: GATGGCACAAGTGCTGAGTGCGATTGTGCCTCCGGCGATG
S5A	A1S8Afw: GCACAATCACGCTCGCCACTTGTGCCATC A1S8Arv: GATGGCACAAGTGGCGAGCGTGATTGTGC
T6A	A1T6Afw: CAATCACGCTCAGCGCTTGTGCCATCTTG A1T6Arv: CAAGATGGCACAAGCGCTGAGCGTGATTG
C7A	A1C7Afw: CAATCACGCTCAGCACTGCTGCCATCTTGAGCAAG A1C7Arv: CTTGCTCAAGATGGCAGCAGTGCTGAGCGTGATTG
S11A	A1S11Afw: GCACTTGTGCCATCTTGGCAAAGCCGTTAGGAAATAAC A1S11Arv: GTTATTTCCTAACGGCTTTGCCAAGATGGCACAAGTGC
C21A	A1C21Afw: GAAATAACGGATACCTGGCTACAGTGACAAAAGAATG A1C21Arv: CATTCTTTTGTCACTGTAGCCAGGTATCCGTTATTTC
T22A	A1T22Afw: GAAATAACGGATACCTGTGTGCAGTGACAAAAGAATGCATG A1T22Arv: CATGCATTCTTTTGTCACTGCACACAGGTATCCGTTATTTC
T24A	A1T24Afw: GATACCTGTGTACAGTGGCAAAAGAATGCATGCC A1T24Arv: GGCATGCATTCTTTTGCCACTGTACACAGGTATC
E26A	A1E26Afw: GTACAGTGACAAAAGCATGCATGCCAAGCTG A1E26Arv: CAGCTTGGCATGCATGCTTTTGTCACTGTAC
C27A	A1C27Afw: GTACAGTGACAAAAGAAGCCATGCCAAGCTGTAAC A1C27Arv: GTTACAGCTTGGCATGGCTTCTTTTGTCACTGTAC
S30A	A1S30Afw: CAAAAGAATGCATGCCAGCCTGTAACTAAGTTCCC A1S30Arv: GGGAACTTAGTTACAGGCTGGCATGCATTCTTTTG
C31A	A1C31Afw: CAAAAGAATGCATGCCAAGCGCTAACTAAGTTCCCAACGCG A1C31Arv: CGCGTTGGGAACTTAGTTAGCGCTTGGCATGCATTCTTTTG

Table 13: List of primers designed on PrimerX web-based software used to introduce the desired mutation on *licA2* peptide, where the first letter represents the aminoacid to be substituted by an alanine residue.

Mutation	Primers (5' → 3')
T1A	A2T1Afw: GACGTCAATCCTGAAGCAACTCCTGCTACAAC A2T1Arv: GTTGTAGCAGGAGTTGCTTCAGGATTGACGTC
T2A	A2T2Afw: GTCAATCCTGAAACAGCTCCTGCTACAACC A2T2Arv: GGTTGTAGCAGGAGCTGTTTCAGGATTGAC
T5A	A2T5Afw: GAAACAACTCCTGCTGCAACCTCTTCTTGG A2T5Arv: CCAAGAAGAGGTTGCAGCAGGAGTTGTTTC
T6A	A2T6Afw: CAACTCCTGCTACAGCCTCTTCTTGGAC A2T6Arv: GTCCAAGAAGAGGCTGTAGCAGGAGTTG
S7A	A2S7Afw: CTCCTGCTACAACCGCTTCTTGGACTTG A2S7Arv: CAAGTCCAAGAAGCGGTTGTAGCAGGAG
S8A	A2S8Afw: CTGCTACAACCTCTGCTTGGACTTGCATC A2S8Arv: GATGCAAGTCCAAGCAGAGGTTGTAGCAG
T10A	A2T10Afw: CAACCTCTTCTTGGGCTTGCATCACAGC A2T10Arv: GCTGTGATGCAAGCCCAAGAAGAGGTTG
C11A	A2C11Afw: CTCTTCTTGGACTGCCATCACAGCCGGTG A2C11Arv: CACCGGCTGTGATGGCAGTCCAAGAAGAG
T13A	A2T13Afw: CTTGGACTTGCATCGCAGCCGGTGTAAC A2T13Arv: GTTACACCGGCTGCGATGCAAGTCCAAG
T17A	A2T17Afw: CACAGCCGGTGTAGCGGTTTCTGCTTC A2T17Arv: GAAGCAGAAACCGCTACACCGGCTGTG
S19A	A2S19Afw: CCGGTGTAACGGTTGCTGCTTCATTATGC A2S19Arv: GCATAATGAAGCAGCAACCGTTACACCGG
S21A	A2S21Afw: GTAACGGTTTCTGCTGCATTATGCCCAACAAC A2S21Arv: GTTGTTGGGCATAATGCAGCAGAAACCGTTAC
C23A	A2C23Afw: CGGTTTCTGCTTCATTAGCCCCAACAACTAAGTGTAC A2C23Arv: GTACACTTAGTTGTTGGGGCTAATGAAGCAGAAACCG
T25A	A2T25Afw: GCTTCATTATGCCCAGCAACTAAGTGTACAAG A2T25Arv: CTTGTACACTTAGTTGCTGGGCATAATGAAGC
T26A	A2T26Afw: CATTATGCCCAACAGCTAAGTGTACAAGCC A2T26Arv: GGCTTGTACACTTAGCTGTTGGGCATAATG
C28A	A2C28Afw: CATTATGCCCAACAACTAAGGCAACAAGCCGATGCTAGGGAATC A2C28Arv: GATTCCCTAGCATCGGCTTGTTGCCTTAGTTGTTGGGCATAATG
T29A	A2T29Afw: CAACAACTAAGTGTGCAAGCCGATGCTAGG A2T29Arv: CCTAGCATCGGCTTGCACACTTAGTTGTTG
S30A	A2S30Afw: CAACAACTAAGTGTACAGCCCGATGCTAGGGAATC A2S30Arv: GATTCCCTAGCATCGGGCTGTACACTTAGTTGTTG
C32A	A2C32Afw: CAACTAAGTGTACAAGCCGAGCCTAGGGAATCATAAAAAATTC A2C32Arv: GAATTTTTTATGATTCCCTAGGCTCGGCTTGTACACTTAGTTG

All the complemented strains constructed were screened for their antagonist capability by colony-bioassay (section 2.4.8, page 61). The production of Bliα and Bliβ peptides was confirmed by LC-ESI-MS (section 2.4.6, page 60) and HR-ESI-MS (4.4.11) analysis of the butanol extract of each strain obtained as described in section 4.4.6.5 (page 112).

4.4.10 Evaluation of *tolC* influence on lichenicidin production

Electrocompetent cells of *E. coli* BW25113 and BW25113Δ*tolC*:*kan* (kindly provided by Prof. Dr. Klaus Hantke, Tübingen) were transformed with the fosmid pLic5 generating the *E. coli* strains BWLic5 and BWΔ*tolC*Lic5, respectively. *E. coli* BWLic5 strain was obtained by

selection with 12.5 μg/mL of Clo and the *E. coli* BWΔ*tolC*Lic5 strain with 12.5 μg/mL of Clo and 50 μg/mL of Kan. Both strains were tested for antibacterial activity by colony-bioassay. Moreover, extracts of both strains prepared as previously described (section 4.4.6.5 on page 112) were analyzed by LC-ESI-MS.

Since no antibacterial activity was observed for the *E. coli* BWΔ*tolC*Lic5 strain and none of the lichenicidin peptides were successfully identified, the reinstatement of *tolC* gene in this strain delivered by a plasmid vector was performed. Therefore, the *tolC* gene was amplified from a bacterial suspension of the *E. coli* BW25113 strain using the primers comp_tolCfw (5'-ACTGCA**TATGAA**GAAATTGCTCCCCATT-3') and comp_tolCrv (5'-TAGAT**CTCGAG**TCAGTTACGGAAAGGGTTATGA-3'), possessing the *Nde*I and *Xho*I recognition sequence (bold). The PCR reaction was performed in a final volume of 50 μL containing 1X of Herculase II reaction buffer, 0.25 mM of dNTP's, 3 pmol/μL of each primer, 1 μL of bacterial suspension and 0.25 μL of Herculase® II fusion DNA polymerase (Agilent Technologies). The parameters of amplification were as follows: 94 ºC for 5 min, 35 cycles of 94 ºC for 30 sec, 54 ºC for 30 sec and 72 ºC for 2 min and a final extension step of 10 min at 72 ºC. Two of such reactions were performed, pooled together and concentrated by final elution with 30 μL of sterile distilled water with the GenJet PCR purification kit (Fermentas). The PCR product was further digested with 10 U of *Nde*I and 20 U of *Xho*I (Fermentas). The reaction contained approximately 1-2 μg of purified PCR product and 1X of Buffer O (Fermentas), in a final volume of 40 μL. The pET15b (Novagen) vector was also digested in the same conditions. The reactions were incubated at 37 ºC for 3 hours, and purified with the GenJet PCR purification kit (Fermentas). Approximately 50 ng of the linear vector was ligated to 150 ng of digested PCR-product in a 20 μL reaction containing approximately 1-2 μg of DNA, 1X ligase buffer and 10 U of T4 DNA ligase (Fermentas). 5 μL of this reaction was used to transform chemically competent *E. coli* DH5α cells and the correspondent transformants selected on LB agar plates containing Amp at 100 μg/mL. Positive clones were screened by colony-PCR using the T7prom (5'-TAATACGACTCACTATAGGGAGACCAC-3') and T7term (5'-CAAAAAACCCCTCAAGACCC-3') primers. Each PCR reaction was performed on a final volume of 12.5 μL containing 1.5 mM of $MgCl_2$, 200 μM of dNTP's, 1X Green GoTaq® Buffer, 0.25 U of GoTaq® Buffer (Promega) and 0.3 pmol/μL of each primer. The amplification parameters were as follows: 94 ºC for 5 min, 35 cycles of 94 ºC for 30 sec, 54 ºC for 30 sec and 72 ºC for 2 min and a final extension of 10 min at 72 ºC. The resulting p*tolC* plasmid was extracted and sequenced to confirm the absence of mutations.

Finally, the p*tolC* plasmid was transformed into electrocompetent *E. coli* BW*ΔtolC*Lic5cells and positive transformants selected on agar plates containing

Amp (100 μg/mL), Kan (50 μg/mL) and Clo (12.5 μg/mL). The obtained strain was designated as *E. coli* BWΔ*tolC*Lic5p*tolC*. The restoration of antibacterial activity was investigated by colony bioassay. Also, its extracts prepared as previously described (section 4.4.6.5, page 112) were analyzed by LC-ESI-MS. The empty pET15-b vector was also transformed into *E. coli* BWΔ*tolC*Lic5to obtain a control strain.

4.4.11 Mass spectrometry analysis

Routine LC-ESI-MS analytics was performed as described on section 2.4.6 (page 60).

The HR-ESI-MS experiments were obtained on an Orbitrap XL mass spectrometer (Thermo Scientific, Bremen, Germany) coupled to an Agilent 1200 HPLC system (Agilent Technologies, Waldbronn, Germany). Separations were performed using an Eclipse XDB C-18 column (Agilent) with a linear solvent gradient (5 % methanol containing 0.1 % of formic acid to 100 % methanol containing 0.1 % of formic acid in 25 min staying 10 min at 100 % methanol).

MS/MS experiments were performed on an Agilent triple quadrupole Mass spectrometer 6460 coupled with an Agilent 1290 HPLC, equipped with Agilent thermostatted column compartment SL+, autosampler and binary pump. The injection of pure compounds was accomplished via loop injection. For extracts, an Agilent column Eclipse plus C18 RRHD was used for separations with a gradient from 5 % to 100 % acetonitrile in 6 min and staying 2 min at 100 % acetonitrile. Fragment voltage and collision energy was optimized for each sample.

4.4.12 Antibacterial activity assays

Antibacterial activity plates, colony and liquid bioassays were performed as described on section 2.4.8 (page 61). For colony bioassays, testing strains were directly streaked into the bioassay plates.

The impact of lichenicidin mutations on bioactivity was determined by an adaptation of the critical dilution assay (Ryan *et al.*, 1996). Briefly, the volume of resuspension of each complemented strain extract was performed in order to normalize the quantity of mutated peptide in solution. The normalization was performed according to peak integration values (considering both double and triple ions) obtained for each peptide from LC-ESI-MS analysis. The extracts used for this analysis were obtained as described in section 3.4.5 on page 78. After the solvent evaporation, the pellets were resuspended in 70 % acetonitrile

accordingly with the obtained peak integration values. Each sample was diluted and dispensed into the wells of bioactivity agar plates. Inhibition zones were evaluated after 18 h incubation at 37 °C. Arbitrary units (AU ml^{-1}) were determined accordingly with the last dilution for which antibacterial activity was visible. Non-producing strains or strains producing low levels of the mutated peptide were not considered for the assay.

CHAPTER V

Lichenicidin self-protection in *Escherichia coli*

Part of this chapter were accepted for publication in the Applied and Environmental Microbiology journal as:

"Lichenicidin biosynthesis in *E. coli*: *licFGEHI* immunity genes are not essential for lantibiotic production and self-protection"

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5.1 Introduction

Lantibiotic's self-protection mechanisms of the Gram-positive producers typically involve an individual immunity protein (LanI) and/or an ABC transporter, usually composed of two or three subunits (LanFE(G)). An ancillary protein for the assembly of a functional ABC transporter has also been described (LanH) (Draper *et al.*, 2008). The presence of the genetic determinants associated with these mechanisms diverge depending on the lantibiotic that is being produced. Strains belonging to genus *Bacillus* are well known to produce several lantibiotics such as subtilin (*B. subtilis* ATCC 6633), mersacidin (*Bacillus* sp. HIL Y-85,54728) and haloduracin (*B. halodurans* C-125) (Banerjee and Hansen, 1988; Chatterjee *et al.*, 1992; McClerren *et al.*, 2006; Lawton *et al.*, 2007). In the class I lantibiotic subtilin, immunity is mediated by the lipoprotein SpaI and the ABC translocator SpaFEG (Stein, 2005). SpaI protein should sequester subtilin at the cytoplasmic membrane level, preventing the accumulation of subtilin molecules, which could lead to pore formation. The ABC transporter SpaFEG complex should function by pumping the lantibiotic from the cell to the environment (Stein *et al.*, 2005; Draper *et al.*, 2008). The self-protection mechanism of the class II lantibiotic mersacidin involves solely the MrsFGE ABC transporter (Guder *et al.*, 2002) and no *lanI*-like gene could be identified in the *mrs* biosynthetic cluster. Despite the lack of experimental data regarding the immunity mechanism of the two-component lantibiotic haloduracin, two sets of LanFEG proteins are encoded within the *hal* gene cluster (Lawton *et al.*, 2007). For other two-component lantibiotics as cytolysin, only CylI protein was associated with a protective effect for the producer strain (Coburn *et al.*, 1999). None of the abovementioned systems require the presence of a LanH protein. In fact, *lanH* genes are absent in the majority of the operons associated with lantibiotics production (Draper *et al.*, 2008). Still, Aso and co-workers (2005)

showed that NukH could cooperate with NukFEG to provide the nukacin ISK-1 immunity. Moreover, it has also been suggested that NukH has the ability to inactivate nukacin ISK-1 after binding it, thus providing immunity in a unique way (Okuda *et al.*, 2005; Draper *et al.*, 2008).

All the machinery necessary for the expression, modification, transport and regulation of the two-component lantibiotic lichenicidin are encoded in 14 contiguous open reading frames found on *B. licheniformis* chromosome. In the previous chapter, the *lic* gene cluster was cloned and successfully expressed in *Escherichia coli* (BLic5 strain). This system was further exploited to investigate the role of all the ORFs constituting the *lic* gene cluster, except those encoding the putative immunity genes. The five polypeptides previously associated with lantibiotic self-protection are putatively encoded within the *lic* biosynthetic cluster by *licFGEHI* ORFs (Rey *et al.*, 2004; Veith *et al.*, 2004). In this chapter, the impact of *licFGEHI* deletion for the lichenicidin self-protection of BLic5 strain was evaluated. Also, the production of the lichenicidin peptides Bliα and Bliβ was investigated in the absence of these ORFs. Moreover, it was experimentally confirmed that Gram-negative OM constitutes a natural barrier that prevents the bactericidal action of lichenicidin peptides on the cytoplasm membrane.

5.2 Results

5.2.1 Deletion of *licFGEHI* genes in *E. coli* BLic5

Although antimicrobial effects of individual peptides Bliα and Bliβ were within the micromolar concentration range, biological activity of their mixture at a 1:1 ratio was shown to be much higher than the sum of their individual contributions (Shenkarev *et al.*, 2010). Thus, Bliα and Bliβ should interact synergistically to exert their antagonist activity against Gram-positive bacteria within the nanomolar concentration range. Taking this information into consideration, the deletion of *licFGEHI* ORFs was firstly performed on the p*Lic5*Δ*A1*and p*Lic5*Δ*A2* fosmids, in which *licA1* and *licA2* structural genes were deleted, respectively to decrease the possibility of bacterial self-killing caused by the synergetic activity of Bliα and Bliβ peptides. The strategy involved the replacement of the five ORFs by an apramycin resistance cassette using the λ RED redirect system as described in the Chapter IV. The constructed fosmids pLic5Δ*A1*Δ*FGEHI:apra* and pLic5Δ*A2*Δ*FGEHI:apra* conferring resistance to chloramphenicol (12.5 μg/mL) and apramycin (50 μg/mL) were transformed into chemically competent *E. coli* BL21 Gold (Stratagene). The obtained strains *E. coli* BLic5Δ*A1*Δ*FGEHI* and *E. coli* BLic5Δ*A2*Δ*FGEHI* were characterized by the absence of bioactivity against the indicator strain *Micrococcus luteus* (Figure 42B/C).

These mutants were also subjected to colony cross-feeding either with Bliα (through the strain BLic5Δ*A2*) or Bliβ (through the strain BLic5Δ*A1*) as described in the previous chapter. Since bioactivity was restored, it was shown that *E. coli* BLic5Δ*A1*Δ*FGEHI* retained the ability to produce Bliβ and *E. coli* BLic5Δ*A2*Δ*FGEHI* to produce Bliα (Figure 43). Such results were also confirmed by LC-ESI-MS analysis of butanol extracts (Figure 42B/C).

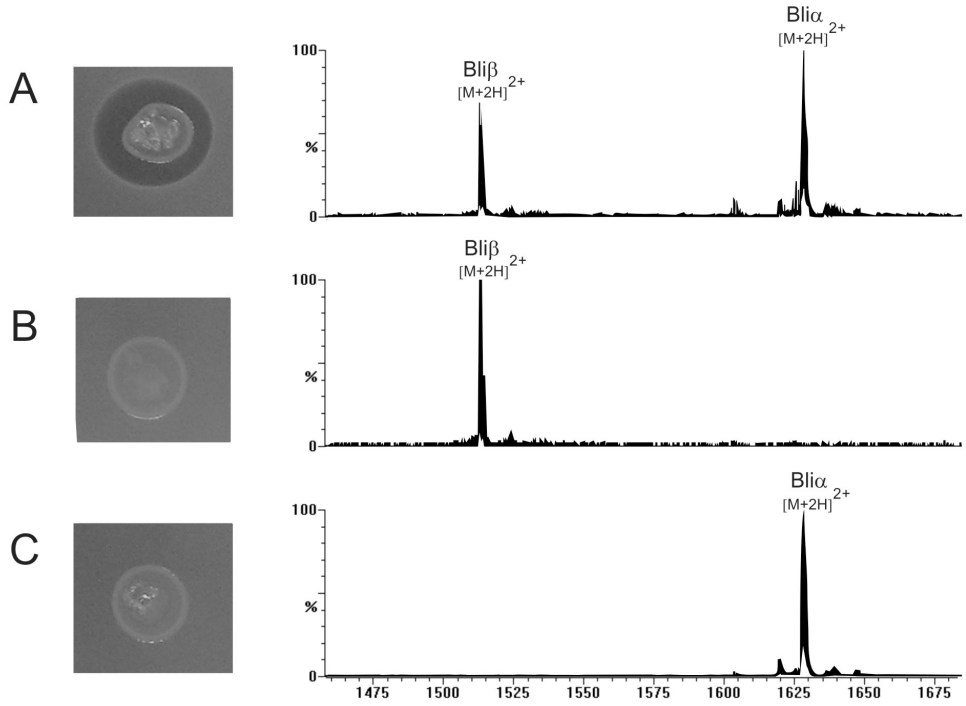

Figure 42: Bioassay of *E. coli* mutants BLic5Δ*FGEHI* (A), BLic5Δ*A1*Δ*FGEHI* (B) and BLic5Δ*A2*Δ*FGEHI* (C) against the indicator strain *M. luteus*. LC-ESI-MS analyses of butanol extracts from the respective mutant liquid culture are also shown.

In order to understand if the bacterial survival of these mutants accompanied by the production of the peptides resulted from Bliα or Bliβ absence, the deletion of *licFGEHI* was attempted in the fosmid p*Lic5* possessing both *licA1* and *licA2* intact structural genes. The resulting plasmid pLic5Δ*FGEHI* was constructed and transformed as abovementioned. Surprisingly, it was possible to obtain *E. coli* BLic5Δ*FGEHI* mutants producing both Bliα and Bliβ peptides, as confirmed by bioassay and LC-ESI-MS analysis (Figure 42A). Subsequently, the susceptibility to lichenicidin of both BLic5 and BLic5Δ*FGEHI* strains was accessed. Agar plates containing each of these strains were prepared the same way as those of the *M. luteus* indicator strain. The lichenicidin sample was obtained from the growth of the BLic5 strain at 37 °C for 24 hours. After overnight incubation, no inhibition zones were

detected for both BLic5 and BLic5Δ*FGEHI* strains around the lichenicidin extract, suggesting that in *E. coli*, the immunity genes are not essential for the lichenicidin self-protection mechanism.

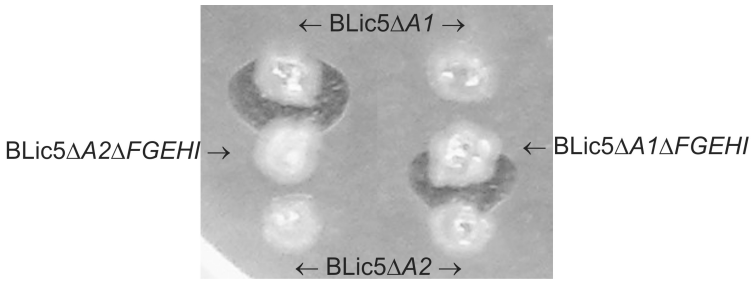

Figure 43: Cross-feeding agar diffusion assay of *E. coli* BLic5Δ*A1*Δ*FGEHI* and BLic5Δ*A2*Δ*FGEHI* mutants with Bliα (BLic5Δ*A2*) and Bliβ (BLic5Δ*A1*) peptides. The bacterial activity restored between BLic5Δ*A1* and BLic5Δ*A2*Δ*FGEHI* suggested that this mutant was able to produce the Bliα peptide. Similarly, the *M. luteus* inhibition zone observed between BLic5Δ*A2* and BLic5Δ*A1*Δ*FGEHI* suggested that this mutant was able to produce the Bliβ peptide.

5.2.2 Lichenicidin production by *E. coli* BLic5 and *E. coli* BLic5Δ*FGEHI*

The abundance of both lichenicidin peptides was investigated by LC-ESI-MS analysis of butanol extracts of BLic5 and BLic5Δ*FGEHI* cultures after 24 hours of growth and performed in triplicate. The values obtained from the TIC (total ion current) peak integration of each peptide were generally higher for BLic5 cultures (Bliα: 585.7±64.0 AU; Bliβ: 236.0±16.6 AU; AU= arbitrary units) than for BLic5Δ*FGEHI* cultures (Bliα: 400.7±169.5 AU; Bliβ: 125.7±41.8 AU) (Figure 44).

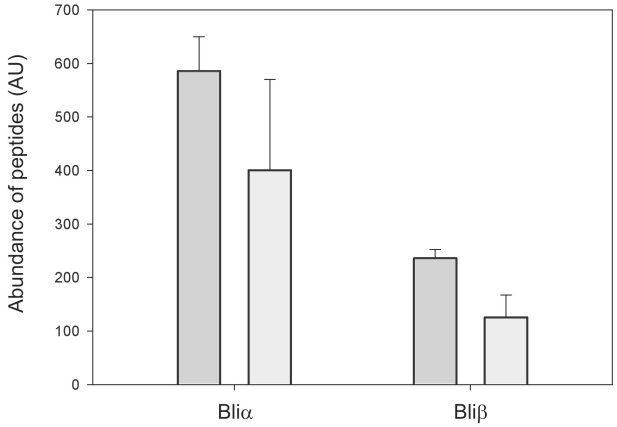

Figure 44: Comparison of Bliα and Bliβ abundance on total butanol extracts of *E. coli* Blic5 (dark green/dark salmon) and *E. coli* Blic5Δ*FGEHI* (light green/light salmon) strains.

5.2.3 Outer membrane and lichenicidin self-protection

It is believed that lichenicidin exerts its activity by inhibiting the cell-wall synthesis followed by the dissipation of the membrane target, similarly to haloduracin and lacticin 3147 (Wiedemann *et al.*, 2006; Oman and van der Donk, 2009b). Thus, its inability to inhibit the Gram-negatives *E. coli* and *Klebiella pneumoniae* (Mendo *et al.*, 2004; Dischinger *et al.*, 2009) is consistent with the presence of an OM, which prevents the diffusion of lantibiotic peptides through the periplasmic space. In this situation, an *E. coli* with permeable OM should be sensible to the extracellular feeding of Bliα and Bliβ peptides mixture. To prove this hypothesis, *E. coli* BLic5 lichenicidin total butanol extracts (containing both Bliα and Bliβ) were dissolved in 70 % ACN/dH_2O and bioassayed against *E. coli imp*4213 and the respective wild-type strain *E. coli* MC4100. *E. coli imp*4213 possesses a mutation in the *imp* gene (*imp*4213), which causes an OM permeability defect (Braun and Silhavy, 2002). Inhibition zones were observed on plates containing the *imp*4213 strain, but not on those containing the MC4100 strain (Figure 45). The activity of each peptide alone on these two strains was also examined. Thus, *E. coli* BLic5Δ*A1* (producing exclusively Bliβ) and *E. coli* BLic5Δ*A2* (producing exclusively Bliα) butanol extracts dissolved in 70 % acetonitrile were bioassayed side by side on plates containing either the *imp*4213 or the MC4100 strain. It was observed that *imp*4213 growth was affected only if both Bliα and Bliβ peptides were present (Figure 45). This suggests that when both Bliα and Bliβ reach the *E. coli* periplasmic space, they can act synergistically on their cellular targets.

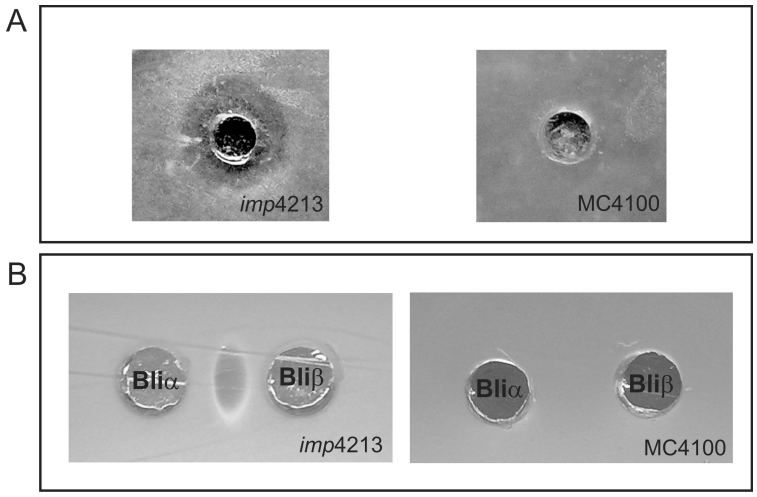

Figure 45: Bioassay of lichenicidin peptides against the *E. coli imp*4213 and MC4100 strains. *E. coli imp*4213 is susceptible to *E. coli* BLic5 cell-free supernatant, containing both Bliα and Bliβ peptides (A). *E. coli imp*4213 growth is only inhibited if both peptides are present (B). The extracts of BLicΔ*A1* and BLicΔA2 strains include only Bliβ or Bliα, respectively (B).

5.2.4 Sub-cellular location of LicP in *E. coli*

The biosynthesis of the mature Bliβ peptide involves two proteolysis steps, similarly to what was described for the Halβ peptide (McClerren *et al.*, 2006). Firstly, the LicA2 leader peptide must be removed by the LicT protein C-terminally from the GlyGly motif, presumably during its translocation through the cytoplasmic membrane. The second step, in the Gram-positive *B. licheniformis* should occur outside the bacterial cell, where the N-terminus of the LicA2 propeptide (NDVNPE) is removed by the extracellular protease LicP, as mentioned in chapter IV. When the *lic* gene cluster was expressed in *E. coli*, the completely processed Bliβ peptide was identified in culture supernatants. Also, the LicP proteolytic activity was detected in culture supernatants of cells expressing the *licP* ORF, but not the other remaining genes of the *lic* biosynthetic cluster. Those results raised the question of whether LicP was being exported to the *E. coli* supernatant or if its extracellular activity was a result of OM leaking (being the periplasm the actual location of LicP). Therefore, the determination the sub-cellular localization of LicP in *E. coli* would be extremely useful for the elucidation of the complete lichenicidin biosynthesis on BLic5 strain. With this in mind, the *E. coli* BLic5Δ*P* strain possessing the deleted *licP* gene was complemented with p*licPHis*$_6$ plasmid to generate the BLic5Δ*PlicPHis* strain. That vector was based on the pET24-a+ plasmid, expressing the LicP protein with a C-terminal His-Tag. The functionality of LicP protein was confirmed by colony bioassay, where the antibacterial activity of BLic5Δ*PlicPHis* against *M. luteus* was restored. The presence of Bliβ peptide in BLic5Δ*PlicPHis* culture supernatants was also confirmed by LC-ESI-MS analysis. Subsequently, liquid cultures of BLic5Δ*PlicPHis* strain were performed. The bacterial cells were precipitated by centrifugation and subsequently divided into their periplasmic and spheroplastic fractions. Finally, the protein profile of these fractions and the bacterial pellet before fractionation was analyzed on SDS-PAGE gels after Comassie Blue staining. The same fractions obtained from BLic5Δ*P* strain cultures were also included in the SDS-PAGE gels for a comparative analysis. With the adopted methodology, no protein bands were visibly detected on samples corresponding to the cultures' supernatants. Regarding the profile obtained for periplasmic and spheroplastic fractions it was found that the first fraction was highly contaminated with cytoplasmic proteins. Moreover, no differences in protein expression were visually detected between the extracts of BLic5Δ*PlicPHis* and BLic5Δ*P* strains. Therefore, in order to detect the LicP protein, a Western-blot analysis with anti-His-Tag antibodies was performed. A positive signal was obtained only with BLic5Δ*PlicPHis* strain samples corresponding to the bacterial pellet before fractionation. The signal was detected between 40 and 55 kDa and it was stronger in the sample resulting from the culture induced with IPTG. In fact, the predicted molecular weight for

LicP was approximately 49 kDa and 46 kDa before and after proteolysis of the six N-terminally located amino acids of LicA2 propeptide, respectively. Thus, these results suggest that, when induced with IPTG, a higher proportion of LicP seems to aggregate into inclusion bodies. Consequently, less biologically active protease would be available for the Bliβ' proteolysis. This result can also explain the decreased antibacterial activity, observed in colony-bioassay in presence of IPTG.

5.3 Discussion

Presently, little information is available with respect to the two-component lantibiotics' immunity systems. Yet, the polypeptides involved in this self-protection mechanism are of particular interest, since it has not been clarified whether they are involved in the protection of the producer cell against one of the two peptides or both (Draper *et al.*, 2008). For lacticin 3147, the best studied two-component lantibiotic, it was demonstrated that LtnI is an essential protein for *Lactococcus lactis* immune phenotype most probably preventing the insertion of the bacteriocins in the membrane or by interacting directly with the peptides, inactivating them (McAuliffe *et al.*, 2000). More recently, Draper *et al.* (2009) showed that both components of the ABC transporter LtnFE are also involved in lacticin 3147 self-protection. In this case, LtnFE should be involved in the export of Ltnα and Ltnβ peptides allowing them to cross the Gram-positive producer strains cell wall (Draper *et al.*, 2009).

In the present study, it was established that in *E. coli*, the expression of the genes involved in the lichenicidin self-protection (*licFGEHI*) of the producer survival is not essential. Moreover, our results demonstrated undoubtedly that *E. coli* cells are able to produce both lichenicidin peptides without the expression of such immunity genes. Yet, it was not clear if this genetic manipulation had a negative impact on the lichenicidin production rate caused for example by a decrease in the bacterial fitness. Although the exact mode of action of lichenicidin peptides was not elucidated so far, it is believed that it would be similar to that of lacticin 3147 (Martin *et al.*, 2004) and haloduracin (Oman and van der Donk, 2009b). This would imply the binding of Bliα to lipid II (a central component in bacterial wall synthesis). Subsequently, the binding of the helical Bliβ to this complex should occur in a 1:1 stoichiometry, promoting the formation of ion-conducting pores in the cytoplasmic membrane, finally leading to cell death (Shenkarev *et al.*, 2010). This model application implies that, in *E. coli,* these peptides can exert their activity only if they are able to reach the periplasmic space. In fact, this was experimentally confirmed in the present study, where an *E. coli* mutant possessing a compromised OM (*imp*4213) had its

growth inhibited by lichenicidin extracts, whereas that was not observed with the wild-type strain with an intact OM. The *imp*4213 mutation has been associated with the susceptibility of *E. coli* to a variety of antibiotics, including vancomycin, which are normally inactive against Gram-negative strains (Ruiz *et al.*, 2005; Wu *et al.*, 2005). Taking all these factors into consideration, lichenicidin self-protection of BLic5 and BLic5Δ*FGEHI* strains should be naturally due to the Gram-negative cell-wall structure, i.e. the presence of an OM, rather than the expression of *licFGEHI* genes. This aspect is also supported by the fact that the growth of BLic5Δ*FGEHI* strain was not susceptible to lichenicidin extracts. In fact, when Bliα and Bliβ are present in the extracellular space, they are not able to cross the OM and thus cannot access their cellular targets. Consequently, the functions that have been atributed to LanFGEHI polypeptides would be dispensable in *E. coli* lichenicidin expressing systems.

As previously reported in chapter IV, the OM-protein TolC seems to be involved in the translocation of the lichenicidin peptides to the extracellular environment. Therefore, Bliα and Bliβ peptides are likely to be exported from the cell by a *sec*-independent type-I transport system. Nonetheless, the exact transport system through the complex Gram-negative cell wall is still under investigation and debate. The results herein presented contribute to the further elucidation of that mechanism. Overall, it was shown that Bliα and Bliβ are active against *E. coli* if they contact with its periplasm. Even so, lichenicidin peptides were still produced by a *licFGEHI*-deficient strain (BLic5Δ*FGEHI*). The association of all these facts strongly suggests that Bliα and Bliβ transport system in *E. coli* should not involve the coexistence of both mature peptides in the periplasmic space. Yet, a periplasmic sublocation of an inactive complex as Bliα and the untrimmed Bliβ' peptide could not be discarded. In such a scenario, LicP protein would be exclusively an extracellular protease. The attempt to identify the LicP location did not retrieve satisfactory results in the course of the present study. Yet, the bacterial system, which can be used in future studies for this purposes was established. The optimization of the concentration of the supernatant samples as well as the cellular fractionation would be determinant for the success of such investigation and would constitute an essential piece for the puzzle of lichenicidin export system in *E. coli*.

5.4 Experimental procedures

5.4.1 Bacterial strains and growth conditions

The *E. coli* MC4100 and MC4100*imp4213* were kindly supplied by Prof. Dr. Silhavy from Princeton University (USA). The strains were grown at 37 ºC and maintained on LB agar plates.

Luria-Bertani (LB; Merck) medium was used for general growth of *E. coli* strains, at 37 ºC or 30 ºC with aeration. Routine antibiotic selection was performed in LB agar plates containing the respective antibiotic concentrations. Medium M (section 2.4.1, page 55) was used for lichenicidin production. *M. luteus* ATCC 9341 was used as the indicator strain to test lichenicidin bioactivity. Also, the protocols for preparation of competent cells, transformation, PCR purification, plasmid extraction and DNA purification from gels are presented in the same section.

5.4.2 Deletion of *licFGEHI* genes

The deletion of *licFGEHI* genes from the fosmids pLic5, pLic5Δ*A1* and pLic5Δ*A2* were performed as described in section 4.4.6, 107. The primers used for the amplification of the apramycin resistance cassette were licFGEHIfw (5'-TGACGTGACAATATCCCGCTTTTGAAAAACCCAAAAGGAGCTAGCATTCCGGGGATCCGT CGACC -3') and licFGEHIrv (5'- AAAGGGAGCCGGGTTTTGCTCAAAGGAGAAAACGGATC GGCTAGCTGTAGGCTGGAGCTGCTTC -3'). The primers used for the screening of the successful substitution of *licFGEHI* genes by the Apra cassette were check_licFfw (5'-CAGCCTTGACGGAAAGGATG-3') and check_liclrv (5'- AGCTTGGCGCAAGCTATCAC-3'). The amplification temperature used for the PCR reactions involving these primers was 52 ºC and the extension time amplification was 4 min. The expected amplicon before and after the substitution of *licFGEHI* genes by the Apra cassette was of approximately 3900 bp and 1500 bp, respectively. In these fosmids, the Apra resistance cassette was not excised.

The final strains BLic5Δ*FGEHI*, BLic5Δ*A1*Δ*FGEHI* and BLic5Δ*A2*Δ*FGEHI* resulted from the transformation of chemically competent *E. coli* BL21 Gold cells with the fosmids pLic5Δ*FGEHI*, pLic5Δ*A1*Δ*FGEHI* and pLic5Δ*A2*Δ*FGEHI*, respectively. Each of these strains was subject to colony bioassay.

5.4.3 Construction of BLic5Δ*P* complementation strain

The *E. coli* BLic5Δ*P* was obtained by the extraction of the pLic5Δ*P* fosmid from the *E. coli*Lic5Δ*P* strain (constructed in chapter IV) according to section 4.4.5 (page 107). For the construction of the p*licP*His plasmid, the *licP* gene was amplified from a bacterial suspension of *B. licheniformis* l89 with the primers comp_licPHis_fw (5'-AAGGAGATATACATATGAAAAGAATATATATTTTTCTC-3') and comp_licPHis_rv (5'-GGTGGTGGTGCTCGAGCTCCTTGTTCATCATTTTCAG-3'). Each PCR reaction was performed in a total volume of 50 μL, containing 1X of Herculase II reaction buffer, 0.25 mM

of dNTP's, 3 pmol/μL of each primer, 1 μL of bacterial suspension and 0.25 μL of Herculase® II fusion DNA polymerase (Agilent Technologies). The parameters of amplification were as follows: 94 ºC for 5 min, 35 cycles of 94 ºC for 30 sec, 50 ºC for 30 sec and 72 ºC for 1 min and a final extension step of 10 min at 72 ºC. Two of such reactions were performed, pooled together and concentrated by final elution with 20 μL of sterile distilled water with the GeneJet PCR purification kit (Fermentas). The plasmid pET24-a(+) was extracted and 5 μg of its DNA were digested in a reaction containing 1 X of FastDigest buffer (Fermentas), 3 μL of FastDigest *Xho*I (Fermentas), 3 μL of FastDigest *Nde*I (Fermentas) in a final volume of 60 μL. The reaction was incubated for 5 min at 37 ºC, purified and concentrated with the GeneJet PCR purification kit (Fermentas) to a final volume of 20 μL. The *licP* PCR product (approximately 1300 bp) was ligated to the digested vector using the In-Fusion Advance PCR cloning kit (Clontech). Each reaction was performed on a final volume of 10 μL containing 100 ng of the linearized vector, 200 ng of the PCR product, 1 X of the In-Fusion reaction buffer and 1 μL of In-Fusion enzyme. The mixture was incubated at 37 ºC for 15 min, followed by 15 min at 50 ºC and then placed on ice. Finally, 40 μL of TE buffer were added. 10 μL of the reaction were used to transform *E. coli* DH5α chemically competent cells. The transformants were selected on LB agar plates containing Kan (50 μg/mL) and the positive clones screened by colony PCR as described in section 4.4.7.1 (page 113). After purification and sequencing the p*licP*His plasmid was transformed into chemically competent *E. coli* BLic5Δ*P* cells. Positive transformants were selected on LB agar plates containing Kan (50 μg/mL) and Clo (12.5 μg/mL). The final complemented strain was designated as BLic5Δ*PlicP*His. Its antagonistic activity was evaluated by colony bioassay. The production of lichenicidin peptides was confirmed by LC-ESI-MS analysis, where total extracts were prepared as described on section 3.4.5 (page 78).

5.4.4 Sub-cellular location of the LicP protease

5.4.4.1 Cell fractionation

A pre-culture of the strains *E. coli* BLic5Δ*PlicPHis*and BLic5Δ*P* was prepared in medium M supplied with the appropriate selective marker. 50 mL of fresh medium without the addition of antibiotics were inoculated with 500 μL of the pre-cultures and grown at 37 ºC with aeration (160 rpm). Two distinct cultures were performed for each strain. When the bacterial density reached the OD_{600nm} of 0.6, one culture of each strain was induced with IPTG at a final concentration of 0.5 mM. 3 mL of the bacterial culture with an OD_{600nm} of 2 were centrifuged for 5 min at 12,000 x g. The pelleted cells were divided into their periplasmic and spheroplastic fractions with the PeriPreps™ Periplasting Kit (Epicentre). The

cells were resuspended with 150 μL of PeriPreps periplasting buffer containing omnicleave endonuclease (Epicentre) by mixing up and down and incubated at room temperature for 5 min. Subsequently, 150 μL of sterile purified water at 4 °C was added, followed by mixing inversion and incubation on ice for 5 min. The cells were again centrifuged at 12000 x g for 2 min at room temperature. The resulting supernatant containing the periplasmic fraction was transferred to a clean microtube and the pellet resuspended with 300 μL of lysis buffer (Epicentre). 3 μL of $MgCl_2$ (1 M) was then added, the solution was mixed by inversion and incubated at room temperature for 5 min. The debris was centrifuged at 12000 x g for 5 min at room temperature and the supernatant containing the spheroplastic fraction was transferred to a new tube. Finally, 6 μL of EDTA (500 mM) were added to this fraction. The 3 mL of culture supernatant were applied to an Amicon Ultra-4Ultracel-30 column (Millipore) and centrifuged at 4,000 x g for 10 min in order to concentrate proteins with a molecular weight above 30 KDa. The concentrated volume constituted the supernatant fraction. Moreover, the pelleted cells of each culture were also resuspended with 300 μL of 1 X SDS-PAGE loading buffer and processed as described in the following section.

The protein content of these four fractions (supernatant, periplasmic, spheroplastic and whole cells) was analyzed by SDS-PAGE and the identification of LicP protein performed by Western-blot.

5.4.4.2 SDS-PAGE analysis

For the protein analysis, the supernatant, periplasmic and spheroplastic fractions were mixed with 5 X SDS PAGE loading buffer at a proportion of 5:1. These samples, together with the previously prepared whole cell samples were boiled at 100 °C for 10 min and applied to the polyacrylamide gel. The running gel was prepared as described in Table 15. The running buffer (1X) was added to the anode and cathode electrodes and the samples were loaded. The electrophoresis was performed 30 min at 50 mA, 30 min at 60 mA followed by 1 hour and 30 min at 80 mA. The protein content was visualized directly in the gels by Comassie staining as described in section 3.4.6 of page 78.

Solution

5 X SDS loading buffer: 1.5 g of SDS, 3.75 mL of 1M Tris (pH 6.8), 0.015 g of bromophenol blue, 7.5 mL of distilled water and 7.5 mL of glycerol. DTT was added to a final concentration of 0.1 M to the sample before boiling.

Table 14: Reagents used for the preparation of monomer solutions of the gels used in this section. APS stands for ammonium persulphate and TEMED for N,N,N',N'-tetramethylethylenediamine. These two compounds should be added immediately prior to pouring the gel. The volumes presented are for the preparation of two gels.

Solutions/Reagents		Stacking gel (4 %)	Separating gel (15 %)
AB-3	(mL)	1.03	5.3
1.5 M Tris-HCl pH 8.8	(mL)	-	5
0.5 M Tris-HCl pH 6.8	(mL)	3.15	-
10% SDS	(µL)	60	200
10% APS	(µL)	40	50
TEMED	(µL)	15	20
Distilled water	(mL)	1.5	9.3
Total volume	(mL)	5.8	20

5.4.4.3 Western-blot analysis

For Western blot analysis, the SDS-PAGE gel was incubated approximately 20 min with the transfer buffer, together with two pieces of blotting paper (Roth; 6 x 9 cm) and the nitrocellulose membrane (Wathman; 6 x 9 cm). After this period the gel was assembled with the membrane and the blotting paper in the Trans-blot SD cell (Bio-Rad) in the following order, starting from the bottom of the apparatus: blotting paper, membrane, gel, blotting paper. The transfer was carried out at 100 mA for approximately 1 hour. Subsequently, the membrane was incubated with the blocking solution for 1 hour at room temperature and with the primary anti-His tag antibody solution at 4 ºC for 16/18 hours. After this period, the membrane was washed three times with 30 mL of TNT buffer containing 1% of BSA. Each of these washes were performed for 20 min and were followed by the incubation of the membrane with 30 mL of the TNT solution containing the 1 % of BSA and 6 µL of the secondary antibody (anti-rabitt-AP; Sigma) at 4º C for 1 hour and 30 min. The membrane was finally washed three times with 30 mL of TNT solution, each time. The membrane was performed in 10 mL of AP buffer containing 300 µL of BCIP (Roche) and 135 µL of NBT (Roche). The membrane was incubated in the dark and the reaction was stopped with distilled water.

Solutions

TNT buffer: 50 mM of Tris/HCl (pH 7.6), 500 mM of NaCl and 0.5 % of Triton X-100.

Transfer buffer: 25 mM of Tris, 195 mM of glycine and 20 % of methanol.

Blocking buffer: TNT buffer containing 3% of BSA.

Primary antibody buffer: TNT buffer containing 1% of BSA and 0.02 % of thimerosal containing 1/1000 of the anti-His tag polyclonal rabbit antibody (Sigma).

AP buffer: 12.1 g of Tris/HCl, 5.8 g of NaCl and 1 g of $MgCl_6.H_2O$ at final pH of 9.5.

BCIP: Preparation of 1 mg/μL solution in DMF.
NBT: Dilution of 6.75 mg in 135 μL of 70 % DMF.

5.4.5 Antagonistic bioassays

The *E. coli* MC4100, *E. coli imp*4213, *E. coli* BLic5 and *E. coli* BLic5Δ*FGEHI* bioassay plates were prepared as those containing *M. luteus* as described in section 2.4.8 (page 61). Likewise, colony-bioassays and liquid samples bioassays were also performed according with the same section.

5.4.6 LC-ESI-MS analysis

The LC-ESI-MS analysis of total extracts were performed as described on section 3.4.6 (page 78).

CHAPTER VI

An approach to lantibiotics engineering on *E. coli*: specificity of lichenicidin biosynthetic machinery

6.1 Introduction

The biological activities of lantibiotics, especially their antibacterial activity, has been of much interest (Cortes *et al.*, 2009). These compounds are a class of ribosomally synthesized peptides that undergo extensive posttranslational modifications (Chatterjee *et al.*, 2005; Willey and van der Donk, 2007). The biosynthetic nature of these compounds represents a key advantage over classical antibiotics (of nonribosomal and polyketide origin) for the development of bioengineering strategies (Field *et al.*, 2010). Therefore, several *in vivo* systems have been developed to express new variants of lantibiotics, either by homologous or heterologous expression systems. Some examples include the class 1 lantibiotics nisin (Dodd *et al.*, 1992; Kuipers *et al.*, 1992) and subtilin (Liu and Hansen, 1992) as well as the class 2 lantibiotics mersacidin (Szekat *et al.*, 2003), nukacin ISK-1 (Aso *et al.*, 2004a) and lacticin 3147 (Cotter *et al.*, 2006a). Studies involving site-directed mutagenesis and more recently random mutagenesis of lantibiotics structural genes have been helpful to establish some guidelines for rational peptide design. Moreover, several peptides with enhanced activity were already obtained as a result of these procedures. Interesting examples include nisin variants active against Gram-negative bacteria (Yuan *et al.*, 2004), mersacidin variants exhibiting twofold increased activity against important pathogens such as MRSA and VRE (Appleyard *et al.*, 2009). Also, the generation of nukacin ISK-1 derivatives which are over produced when compared with the wild-type peptide (Islam *et al.*, 2009). Some of these studies also showed that the biosynthetic enzymes for both class 1 and 2 lantibiotics can tolerate several amino acid changes in the respective prepropeptides (Patton *et al.*, 2008). Even so, some of such alterations did not yield the desrired peptide variant at all. Under these circumstances it

was not clear if the modifications were not compatible with the host biosynthetic, transport and/or immunity system (Cortes *et al.*, 2009; Field *et al.*, 2010). Therefore, the recognition of the catalytic flexibility as well as the nature of the enzymatic interactions of the other Lan proteins, apart from LanA prepropeptides, became a prerequisite for the bioengineering of lanbiotics. In this context, the recognition of the *in vitro* activity of some of these enzymes, especially LanM, LanB and LanC greatly contributed to the knowledge in this field.

For class 2 lantibiotics, the *in vitro* reconstitution of the lacticin 481 modifying-enzyme LctM revealed its permissive substrate specificity (Xie *et al.*, 2004). The importance of such characteristics in peptide engineering was already proved by the production of synthetic non-lantibiotic peptides containing both natural and non-proteinogenic amino acids (Levengood *et al.*, 2009). In addition, LctM enzyme was able to process the lacticin 481-like lantibiotics nukacin ISK-1, mutacin II and ruminococcin A to active compounds through the fusion of their propeptides with the LctA leader sequence (Patton *et al.*, 2008). The same study proved that LctM was capable of processing the LctA propeptide N-terminally fused with the MutA (mutacin II prepropeptide) and NukA (nukacin ISK-1) leader sequences (Patton *et al.*, 2008). These findings were in agreement with those *in vivo* experiments conducted for the class 1 lantibiotics nisin and subtilin (Kuipers *et al.*, 1993b; Chakicherla and Hansen, 1995). To the best of our knowledge, similar studies for class 2 lantibiotics were performed with the nukacin ISK-1 and lacticin 3147 whole-cell expression systems (Aso *et al.*, 2004a; Nagao *et al.*, 2007; Kuipers *et al.*, 2008).

Equally important are the LanT biosynthetic enzymes of class 2 lantibiotics, comprising the N-terminal peptidase domain and the C-terminal ATP-binding domain (Chatterjee *et al.*, 2005). Their bifunctional activity involves the removal the leader sequence as well as the transport of the lantibiotic peptides. *In vivo* studies with lacticin 3147 (Kuipers *et al.*, 2008) and lacticin 481 (Uguen *et al.*, 2005) biosynthetic machineries indicated that both LtnT and LctT exhibit relaxed substrate specificity. Nonetheless, the presence of the double glycine domain cleavage site (GG/GA) was always found to be indispensable for the leader sequence removal (Uguen *et al.*, 2005). This was also indispensable for the catalytic process of the LctT protease domain involving *in vitro* assays (Ihnken *et al.*, 2008).

In chapter IV a heterologous expression system was developed for the class II lantibiotic lichenicidin. With that system some lichenicidin variants were produced for the first time, through a *trans* complementation approach. In this chapter the same strategy was employed to investigate the flexibility of the lichenicidin machinery to interact with the haloduracin biosynthetic elements in the heterologous host *E. coli*.

6.2 Results

6.2.1 Complementation of lichenicidin mutants with haloduracin-biosynthetic genes

Haloduracin HalA1 and HalA2 propeptides are highly related to the prepropeptides LicA1 and LicA2, with homologies of 46 % and 44 %, respectively. However, if the analysis is narrowed to their leader sequences, lower similarities were observed, being 31 % between LicA1 and HalA1 (Figure 46A) and 22 % between LicA2 and HalA2 (Figure 46B). Regarding propeptides sequences, LicA1 and HalA1 and LicA2 and HalA2 share a similarity of 47 % and 56 %, respectively (Figure 47). This is mostly due to the conserved amino acid residues located at the C-terminus. Beholding the analysis of LicM modifying enzymes, the highest similarity scores were obtained with HalM1 (35 % homology) and HalM2 (35 % homology). The lichenicidin transporter/peptidase LicT shared the highest homology (50 %) with its counterpart involved in the haloduracin biosynthetic pathway, HalT. Thus, the polypeptides and enzymes intervening in haloduracin production were found to be more related to those intervening in the lichenicidin synthesis.

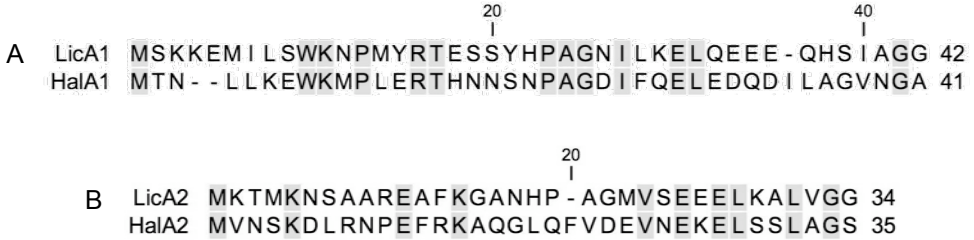

Figure 46: Alignment of the leader sequences of lichenicidin (LicA1 and LicA2) and haloduracin (HalA1 and HalA2) prepropeptides. The conserved residues between the two sequences are highlighted in grey.

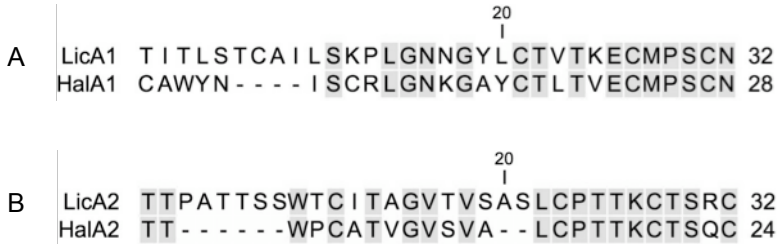

Figure 47: Alignment of the lichenicidin (LicA1 and LicA2) and haloduracin (HalA1 and HalA2) propeptides. The conserved residues between the two sequences are highlighted in grey.

Based on these findings, *E. coli* BLic5 knockout mutants lacking each of the abovementioned *lic* genes were complemented with its counterpart of the haloduracin

biosynthetic operon. For that, competent cells of *E. coli* BLic5Δ*A1*, BLic5Δ*A2*, BLic5Δ*M1*, BLic5Δ*M2* and BLic5Δ*T* strains were transformed with the plasmids p*halA1*, p*halA2*, p*halM1*, p*halM2* and p*halT*, respectively to obtain the BLic5Δ*A1*p*halA1*, BLic5Δ*A2*p*halA2*, BLic5Δ*M1*p*halM1*, BLic5Δ*M2*p*halM2* and BLic5Δ*T*p*halT* complemented strains (Table 15). The same approach was used for plasmids encoding the *licA1*, *licA2*, *licM1*, *licM2* and *licT* genes to obtain the positive controls for this experiment (Table 15). Subsequently, all the constructed strains were bioassayed against *M. luteus* and LC-ESI-MS analysis of extracts recovered from medium M cultures (hereafter referred as "extracts") was performed.

As established in chapter IV, the deletion of the genes encoding the lichenicidin prepropeptides (*licA1* or *licA2*), the modifying enzymes (*licM1* or *licM2*) and the dual transporter/protease (*licT*) resulted in the abolishment of visible *M. luteus* growth inhibition. That was a consequence of the abrogation of the production of either Bliα or Bliβ (except for Δ*T*, where the synthesis of both peptides was abolished). Thus, the complementation of these mutants with each of the p*licA1*, p*licA2*, p*licM1*, p*licM2* and p*licT* plasmids should result on strains with restored biosynthesis of the lichenicidin peptides and consequently possessing antagonistic activity. Indeed, that was observed in the present study, where the molecular masses of both Bliα (M= 3250 Da) and Bliβ (M=3020 Da) peptides were always detected in the *lic* complementation strains extracts (Table 15). Regarding the complementation of the same knockout mutants with the haloduracin genetic determinants, restoration of colony-bioactivity was only observed for the BLic5*ΔT*p*halT* strain, indicating that both peptides Bliα and Bliβ were synthesized (Table 15). Actually, the LC-ESI-MS analysis of BLic5Δ*T*p*halT* extracts revealed the presence of both peptides in its bacterial supernatant. The analysis of BLic5Δ*A1*p*halA1* and BLic5Δ*A2*p*halA2* strains implied that lichenicidin-modifying enzymes could process the lichenicidin prepropeptides and therefore the Halα/Bliβ and Bliα/Halβ peptides' complexes, respectively, would be produced. However, only the molecular masses of Bliβ and Bliα peptides in the extracts of these two bacterial strains were identified (Table 15). The Halα (M= 2331 Da) and Halβ (M= 3043 Da) peptides or some of their derivatives (with different levels of dehydration or proteolysis) could not be detected in the same extracts.

The ability of haloduracin modifying enzymes HalM1 and HalM2 to replace the LicM1 and LicM2 functionality was tested by the construction of BLic5Δ*M1*p*halM1* and BLic5Δ*M2*p*halM2* strains. The LC-ESI-MS analysis of their extracts revealed that BLic5Δ*M1*p*halM1* and BLic5Δ*M2*p*halM2* produced only Bliβ and Bliα peptides, respectively (Table 15). Consequently, none of the lichenicidin peptides (or their derivatives) whose biosynthetic pathway was initially disrupted could be identified.

Table 15: Results obtained for the *E coli* BLic5 knockout mutants complemented with haloduracin biosynthetic determinants, regarding their antagonistic activity as well as the results of LC-ESI-MS analysis. + and – symbols represent the presence or absence of antibacterial activity of the complemented strain against *M. luteus*. The genes which were allocated in the pET24-a(+) vector in the complementation strains are highlighted with bold characters.

Strain	*lanA*		*lanM*		*lanT*	Colony bioassay	MS outcome (Da) Expected	Outcome
BLic5ΔA1p*licA1*	***licA1***	*licA2*	*licM1*	*licM2*	*licT*	+	3250 and 3020	3250 and 3020
BLic5ΔA2p*licA2*	*licA1*	***licA2***	*licM1*	*licM2*	*licT*	+	3250 and 3020	3250 and 3020
BLic5ΔM1p*licM1*	*licA1*	*licA2*	***licM1***	*licM2*	*licT*	+	3250 and 3020	3250 and 3020
BLic5ΔM2p*licM2*	*licA1*	*licA2*	*licM1*	***licM2***	*licT*	+	3250 and 3020	3250 and 3020
BLic5ΔTp*licT*	*licA1*	*licA2*	*licM1*	*licM2*	***licT***	+	3250 and 3020	3250 and 3020
BLic5ΔA1p*halA1*	***halA1***	*licA2*	*licM1*	*licM2*	*licT*	-	2331 and 3020	3020
BLic5ΔA2p*halA2*	*licA1*	***halA2***	*licM1*	*licM2*	*licT*	-	3250 and 3043	3250
BLic5ΔM1p*halM1*	*licA1*	*licA2*	***halM1***	*licM2*	*licT*	-	3250 and 3020	3020
BLic5ΔM2p*halM2*	*licA1*	*licA2*	*licM1*	***halM2***	*licT*	-	3250 and 3020	3250
BLic5ΔTp*halT*	*licA1*	*licA2*	*licM1*	*licM2*	***halT***	+	3250 and 3020	3020 and 3250

6.2.2 Production of haloduracin peptides directed by lichenicidin leader sequences

As shown in the last section, the *in vivo* production of haloduracin peptides using the lichenicidin biosynthetic machinery, directed by *halA1* and *halA2* structural genes was not successful. The leader sequences have been described as important factors for the correct dehydration and cyclization of lantibiotics' propeptides. With that in mind, the synthesis of haloduracin bioactive peptides directed by LicA1 and LicA2 leader sequences was attempted. To accomplish this objective, two genes encoding the chimeric peptides $LicA1_{L}HalA1_{P}$ and $LicA2_{L}HalA2_{P}$ (Figure 48) were synthetically produced. Their respective ORFs were further cloned on the pET24-a(+) vector to obtain the plasmids p*chimeraA1* and p*chimeraA2*. Subsequently, these two plasmids were transformed into BLic5*ΔA1* and BLic5*ΔA2* knockout mutants to originate the BLic5*ΔA1*p*chimA1* and BLic5*ΔA2*p*chimA2* strains, respectively. Finally, these two strains were bioassayed against *M. luteus* and their extracts obtained from fermentations in medium M cultures were further analyzed by LC-ESI-MS.

It was observed that both strains were not able to inhibit *M. luteus* growth. However, antagonistic activity was observed when BLic5Δ*A1*p*chimA1* was incubated next to BLic5Δ*A2* and BLic5Δ*A2*p*chimA2* next to BLic5Δ*A1*, suggesting the production of Bliβ by BLic5Δ*A1*p*chimA1* and Bliα by BLic5Δ*A2*p*chimA2* strains. In fact, the molecular masses of

Bliβ and Bliα were successfully detected on the extracts of BLic5Δ*A1*p*chimA1* and BLic5Δ*A2*p*chimA2*, respectively. None of the haloduracin peptides or some of their derivatives could be identified (Table 16).

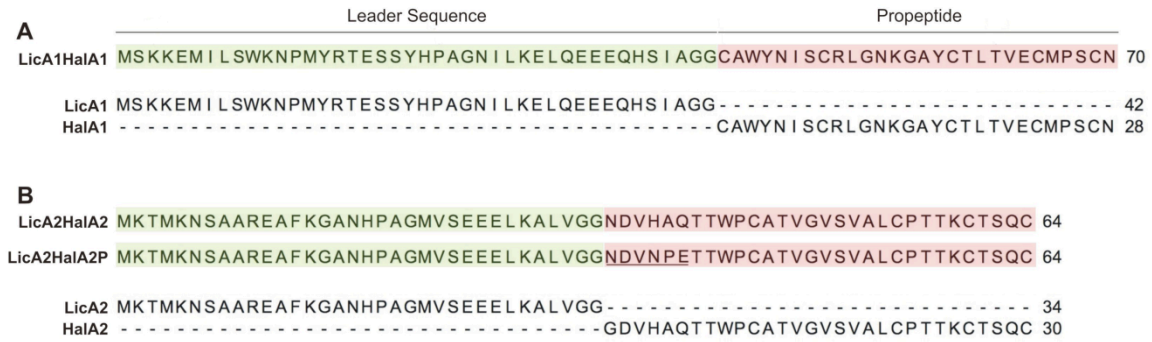

Figure 48: Amino acid sequence of LicA1$_L$HalA1$_P$ (A), LicA2$_L$HalA2 $_P$ and LicA2$_L$-NDVNPE-HalA2 $_P$ (B) chimeric peptides encoded by p*chimeraA1*, p*chimeraA2* and p*chimeraA2.1*, respectively. The leader sequence of lichenicidin peptides (tagged as LicA1 and LicA2) and the haloduracin propeptides sequences (tagged as HalA1 and HalA2) are also represented.

As discussed in chapter IV, the production of Bliβ and Halβ involves a proteolysis step after the leader peptide processing involving the removal of the six N-terminally located amino acids of their propeptides. LicP is the protease involved in this reaction for the Bliβ peptide (chapter IV). However, its counterpart in the Halβ biosynthesis was not described so far. The untrimmed Bliβ' and Halβ' correspond to the NDVNPE-Bliβ and GDVHAQ-Halβ peptides, respectively. Thus, the conservation identified in the Bliβ' and Halβ' N-termini is restricted to Asp-5 and Asn-6 residues. The *licA2$_L$halA2$_P$* ORF was first designed to originate a chimeric peptide containing the Halβ' sequence in the C-terminal of the LicA2 leader sequence (Figure 48). Nevertheless, no information is available concerning the specificity of LicP protease so far. Therefore, the absence of the Halβ peptide in the BLic5Δ*A2*p*chimA2* extracts could have been due to a failure of the trimming of the Halβ' N-terminal by the LicP protease. In this context, a new ORF was obtained by site-directed mutagenesis, whose expression resulted in the chimeric peptide LicA2$_L$-NDVNPE-HalA2$_P$. In this peptide the N-terminal of Bliβ', which is believe to be involved in the LicP catalytic specificity, substituted that of the Halβ' peptide (Figure 48). The plasmid containing the new ORF (p*chimeraA2.1*) was used to transform the BLic5Δ*A2* knockout mutant, originating the BLic5Δ*A2*p*chimA2.1* strain. Even so, this new strain was still unable to inhibit the growth

of *M. luteus*. Also, the molecular masses of Halβ peptide or their derivatives could not be identified by LC-ESI-MS analysis of its extracts, where Bliα peptide was detected (Table 16).

Table 16: Results obtained for the *E coli* BLic5 knockout mutants complemented with the lichenicidin/haloduracin chimeric genes. The genes encoding the lichenicidin modifying enzymes and its transporter/protease were maintained in all the strains. - symbols represents the absence of antagonistic activity. The molecular masses of 2331 Da and 3043 Da correspond to the fully modified Halα and Halβ peptides. [a)] This mass takes in account that LicP is able to remove the N-terminal of Halβ' peptide.

Strain	*lanA*		Colony bioactivity	MS outcome (Da)	
				Expected	Detected
BLic5Δ*A1pchimeraA1*	***licA1halA1***	*licA2*	-	2331 and 3020	3020
BLic5Δ*A2pchimeraA2*	*licA1*	***licA2halA2***	-	3043[a)] and 3250	3250
BLic5Δ*A2pchimeraA2P*	*licA1*	***licA2PhalA2***	-	3043 and 3250	3250

6.3 Discussion

The results herein presented revealed that the transporter/protease present in the haloduracin gene cluster (*halT*) is able to transport and remove the leader sequence of LicA1 and LicA2 prepropeptides when the peptides are expressed in *E. coli*. Previous studies have already demonstrated the promiscuity of LtnT *in vivo*, since nisin and angiotensin peptides fused with LctA2 leader sequence were successfully transported and proteolytic processed by *Lactococcus lactis* (Kuipers *et al.*, 2008). In our study, no such fusions were made. Thus, our results suggest that the absence of HalA1 and HalA2 leader sequences were not critical for the export of Bliα and Bliβ peptides by HalT. Still, it is important to emphasize that both lichenicidin and haloduracin peptides possess the double glycine motif (GG/GA) N-terminally of their propeptide sequences. According to previous studies (Uguen *et al.*, 2005; Ihnken *et al.*, 2008), we believe that these motifs are essential for the removal of LicA1 and LicA2 leader sequences by HalT. Similarly, Nagao and co-workers (2007) demonstrated that the lacticin 481 LctT was able to replace the function of nukacin ISK-1 NukT in the transport and leader sequence proteolysis of NukA in a whole-cell system.

The replacement of *licA1* and *lic*A2 by the structural genes of the haloduracin gene cluster were performed to investigate the dehydratation and cyclization ability of LicM1 and LicM2 of the haloduracin prepropeptides. The results were inconclusive, since molecular masses of Halα and Halβ or their derivatives (e.g. peptides with different levels of dehydration) could not be detected. Therefore, it was not possible to understand if LicM1 and LicM2 were completely unable to process haloduracin prepropeptides or alternatively

if the host could not cope with a non-fully modified Halα and Halβ peptide. However, regarding the HalA2 propeptide it is important to refer that the protease responsible for Halβ second proteolysis step was not present and it was not possible to established if the LicP protease is able to process the Halβ' N-terminus. Similar results were herein obtained in the experiments where the ability of haloduracin modifying enzymes HalM1 and HalM2 to process lichenicidin prepropeptides was evaluated. In this situation, *halM1* was expressed in a *licM1*-deficient strain and *halM2* in a *licM2*-deficient strain. LC-ESI-MS analysis revealed that these strains were unable to produce Bliα and Bliβ peptides, respectively, or any of its derivatives. Thus, it was also not possible to establish if the HalM1 and HalM2 processing of lichenicidin propeptides were completely or only partially inefficient. In fact, *in vivo* experiments on both class 1 and class 2 lantibiotics have demonstrated that the modifying enzyme are not able to process prepropeptides from distinct (even closely-related) biosynthetic pathways. For instance, when the complete lacticin 481 prepropeptide was expressed in a nukacin ISK-1-producer cell, no lacticin 481-related peptide products were produced (Nagao *et al.*, 2007). The same results were obtained for class 1 lantibiotics, since the *B. subtilis* subtilin-production machinery was not able to process the nisin prepropeptide (Rintala *et al.*, 1993). Also, no nukacin ISK-1 derivates were identified when the LctM enzyme replaced the function of NukM in the modification of NukA prepropeptide (Nagao *et al.*, 2007). On the contrary, *in vitro* assays demonstrated that the LctM enzyme is able to process NukA substrate into a 4-fold dehydrated peptide, which was biologically active after proteolytic removal of its leader sequence (Patton *et al.*, 2008).

Considering the class 1 lantibiotics subtilin and nisin, it was also hypothesized that subtilin machinery was incapable to process the nisin prepropeptide, since its gene products were not detected (Rintala *et al.*, 1993). However, when the subtilin leader sequence was fused with the nisin propeptide sequence, a dehydrated but inactive product was produced by the subtilin biosynthetic system (Chakicherla and Hansen, 1995). Moreover, chimeras of lacticin 3147 leader sequence and forms of angiotensin peptides were modified, processed and transported by LtnM2 and LtnT in the *L. lactis* host (Kuipers *et al.*, 2008). The same capability was demonstrated for the LctM enzyme, in *in vitro* assays, since it was able to catalyze the dehydration and cyclization of nukacin ISK-1, mutacin II and ruminococcin A propeptides fused with the LctA leader sequence (Patton *et al.*, 2008). To clarify this aspect, the ability of lichenicidin biosynthetic machinery to cope with Lic_L-Hal_P chimeras was also evaluated. To accomplish this, nucleotide sequences corresponding to LicA1 and LicA2 leader sequences were fused with those corresponding to HalA1 and HalA2 structure peptides, respectively. After expression of $LicA1_L$-$HalA1_P$ and $LicA2_L$-$HalA2_P$

chimeras with the lichenicidin machinery, no haloduracin peptides or its derivatives were identified by LC-MS-ESI analysis. The influence of the six N-terminal amino acids that are trimmed from Bliβ' and Halβ' peptides to produce the fully active forms was also evaluated. The LicA2$_L$-HalA2$_P$ chimera possessed the Halβ' N-terminus, whereas in LicA2$_L$-NDVNPE-HalA2$_P$ chimera the equivalent sequence of Bliβ' was introduced. Still, in the presence of a substrate for the LicP protease, it was not possible to identify the Halβ peptide or its derivatives. As such we proved that these results were not affected by the action of LicT. So, it can be expected that even in the presence of the lichenicidin leader sequences, the LicM1 and LicM2 dehydration and cyclization of haloduracin is not completely efficient. It is believed that LanM enzymes process their peptide subtrates distributively and directionally, moving from the N- to the C-terminus (Lee *et al.*, 2009; Oman and van der Donk, 2009a). However, the conservation of the N-terminus of lichenicidin and haloduracin propeptides is very low (Figure 47). Such differences are possibly preventing the correct dehydratation and ring formation of haloduracin propeptides. In fact, this could explain the unsuccessful identification of Halα and Halβ characteristic molecular masses as well as that of their derivatives. For instance, in chapter IV it was found that as a result of the perturbation of C-terminally located thioether rings in the Bliα and Bliβ peptides, the mutated peptide(s) or its derivatives were not detected by LC-ESI-MS analysis. Similar results were obtained for lacticin 3147 Ltnα and Ltnβ variants (Cotter *et al.*, 2006a). We believe that the same tendency should be expected for the *in vivo* biosynthesis of haloduracin, especially considering the conservation of C-terminally located amino acids and ring structures between the lichenicidin and haloduracin mature peptides (Figure 32, page 85). According to this, a distortion in the Halα or Halβ C-terminal cyclization pattern would surely prevent their identification in the bacterial extracts.

Further information provided by this system will help to establish the specificity of the lichenicidin biosynthetic enzymes. The exploitation of the BLic5 expression system will also allow the generation of other lantibiotics, including those that were not previously characterized from their primary producers. Indeed, a recent study demonstrated that nisin biosynthetic machinery can produce active forms of the PneA1 and PneA2 propeptides, which represents a two-component lantibiotic encoded in the chromosome of *Streptococcus pneumoniae* R6 strain (Majchrzykiewicz *et al.*, 2010).

6.4 Experimental procedures

The lichenicidin and haloduracin genes were amplified from *Bacillus licheniformis* I89 and *Bacillus halodurans* C-145, respectively. The amplification reactions were performed in

a final volume of 25 μL, containing 1X of Herculase® II reaction buffer, 0.25 mM of dNTP's, 0.3 pmol/μL of each primer and 0.25 μL of Herculase® II fusion DNA polymerase (Agilent Technologies). Bacterial suspensions were prepared in 100 μL of distilled water and 1 μL of this solution was used as the DNA template for the reaction. The suspension of *B. halodurans* C-145 was previously incubated at 100 ºC for 10 min. Each of these reactions was performed in quadriplicate. The amplifications parameters were as follows: 1 cycle at 95 ºC for 2 min, 30 cycles of 95 ºC for 20 sec, primer annealing temperature for 20 sec and 72 ºC for the required extension time and a final step of 72 ºC for 3 min. The annealing temperature and extension time were dependent on the gene to be amplified and are described in Table 17. The primers used for the amplifications were designed to include two different restriction sites and are described in Table 18. After the amplification, the four independent reactions performed for each gene were pooled together, purified with the GeneJet PCR purification Kit (Fermentas) and eluted in 25 μL of sterile distilled water. Each purified PCR products was digested with the restriction enzymes listed on Table 18. Each reaction was performed according with the conditions described in the web-based software DoubleDigest (http://www.fermentas.com/en/tools/doubledigest) from Fermentas. After purification and concentration of the digestion reactions with the GeneJet PCR purification kit, the PCR fragments were ligated to the previously digested vector pET24-a(+), according to the T4 DNA ligase (Fermentas) suppliers' instructions. 5 μL of the ligation reaction were used to transform chemically competent *E. coli* DH5α cells and the transformants were selected on LB agar plates containing Kan (50 μg/mL). Positive clones were screened by colony-PCR with T7prom and T7term primers as described in section 4.4.7.1 (page 113) applying the extension times listed on Table 17 for amplification. Finally, the constructed plasmids were extracted with the GeneJet plasmid extraction Kit (Fermentas) and sequenced to confirm the absence of mutations.

Table 17: Parameters used for the amplification of lichenicidin and haloduracin biosynthetic genes. The size of the expected amplicons is also included.

Target gene	Amplification parameters		Expected amplicons
	Annealing temperature	Extension time	
licM1	52 ºC	3 min	3181 bp
licM2	52 ºC	3 min	3103 bp
halA1	52 ºC	45 sec	226 bp
halA2	51 ºC	45 sec	219 bp
halM1	50 ºC	3 min	3180 bp
halM2	50 ºC	3 min	3010 bp
licA1$_L$-halA1$_P$	52 ºC	45 sec	219 bp
licA2$_L$-halA2$_P$	51 ºC	45 sec	201 bp

Table 18: Sequence of the primers used for the amplification of lichenicidin and haloduracin biosynthetic genes. The restriction enzymes used are also represented; the recognition sequences are highlighted in bold in the respective primer sequence.

Target	Primer		Restriction Enzyme
	Designation	Sequence (5' → 3')	
licM1	Comp_licM1fw	AGGTC**GGATCC**ATGAATGAAAAATCC	*Bam*HI
	Comp_licM1rv	CATAGATT**CTCGAG**TTAAAACACGTTTTC	*Xho*I
licM2	Comp_licM2fw	ATCA**GGATCC**ATGAGCATGAAAGAATTCG	*Bam*HI
	Comp_licM2rv	TAGT**GCGGCCGC**TCACCTGCCCGTCGGAATATC	*Not*I
halA1	Comp_halA1fw	ATATA**CATATG**ACAAATCTTTTAAAAG	*Nde*I
	Comp_halA1rv	TAGAT**CTCGAG**TTAGTTGCAAGAAGGCATGCACTC	*Xho*I
halA2	Comp_halA2fw	ATATA**CATATG**GTAAATTCAAAAGAT	*Nde*I
	Comp_halA2rv	TAGAT**CTCGAG**TTAGCACTGGCTTGTACAC	*Xho*I
halM1	Comp_halM1fw	ATCA**AAGCTT**TAATGAGAGAATTACAAAATGCTCTTTAC	*Hind*III
	Comp_halM1rv	TAGAT**CTCGAG**ATGATGTTCATTGTTAATG	*Xho*I
halM2	Comp_halM2fw	ATCA**GGATCC**ATGAAAACTCCTCTAAC	*Bam*HI
	Comp_halM2rv	TAGAT**CTCGAG**TCTATGTTTGATCTTATTATC	*Xho*I

6.4.1 Construction of the complementation strains

The fosmids pLic5Δ*M1*, pLic5Δ*M2* and pLic5Δ*T* obtained in chapter IV were isolated from the corresponding *E. coli* EPI3000 cells as described in section 4.4.5 (page 107). 2 μL of each plasmid were used to transform chemically competent *E. coli* BL21 Gold cells, to obtain the strains BLic5Δ*M1*, BLic5Δ*M2* and BLic5Δ*T* respectively. Positive transformants were selected on LB agar plates containing Clo (12.5 μg/mL). Finally, the plasmids encoding the lichenicidin and haloduracin modifying enzymes and transporter/protease were transformed into each of the following BLic5 knockout mutants: i) p*LicM1* and p*HalM1* into BLic5Δ*M1*, ii) p*LicM2* and p*HalM2* into BLic5Δ*M2* and iii) p*LicT* and p*HalT* into BLic5Δ*T*. The plasmids encoding the structural haloduracin genes p*HalA1* and p*HalA2* were transformed into the BLic5Δ*A1* and BLic5Δ*A2* strains obtained in chapter IV. Positive transformants were selected on LB agar plates containing Clo (12.5 μg/mL) and Kan (50 μg/mL).

6.4.2 Construction of BLic5 strains expressing lichenicidin-haloduracin chimeras

The genes encoding the chimeras LicA1$_L$-HalA1$_P$ and LicA2$_L$-HalA2$_P$ were synthesized and provided in the pMA-T vector by GENEARTE company (Germany). Subsequently LicA1$_L$-HalA1$_P$ and LicA2$_L$-HalA2$_P$ chimeras were amplified using the primers comp_licA1fw and comp_halA1rv (Table 11, chapter IV and Table 18) and comp_licA2fw/comp_halA2rv

(Table 11, and Table 18), respectively. After the PCR amplification, the amplicons were digested with the appropriate restriction enzymes (Table 18 and section 4.4.8) and ligated to pET24-a(+) vector according to the manufacturer's instructions. The ligation was transformed into chemically competent DH5α cells and positive transformants were selected on LB agar plates containing Clo (12.5 μg/mL) and Kan (50 μg/mL). After colony-PCR screening with the T7prom and T7term primers (section 4.4.7.1, page 113) the inserts of the constructed plasmids p*chimeraA1* and p*chimeraA2* were sequenced prior to further procedures. The p*chimeraA2.1* plasmid was obtained by site-directed mutagenesis as previously described in 4.4.9 (page 115). The primers used for amplification were chimeraA2fw (5'-GGTAGGAGGAAATGATGTGAATCCGGAAACAACTTGGCCTTGCG-3') and chimera A2rv (5'-CGCAAGGCCAAGTTGTTTCCGGATTCACATCATTTCCTCCTACC-3'). Finally, p*chimeraA1* was transformed into BLic5Δ*A1* chemically competent cells whereas p*chimeraA2* and p*chimeraA2.1* was transformed into BLic5Δ*A2* chemically competent cells. Positive transformants were selected on LB agar plates containing Clo (12.5 μg/mL) and Kan (50 μg/mL).

6.4.3 Antagonistic activity bioassay and LC-ESI-MS analysis

The antagonistic activity bioassays were performed against *M. luteus* as previously described in section 2.4.8 (page 61). Peptides were recovered from liquid cultures, after butanol extraction (see sections 3.4.5 and 4.4.6.5) and were further analysed by LC-ESI-MS as described in section 2.4.6 (page 60).

CHAPTER VII

Phylogenetic analysis of NRPSs from Portuguese lakes and caves

7.1 Introduction

The recognition of rRNA genes as evolutionary chronometers and their analysis for the description of environmental microorganisms diversity without culturing techniques completely changed the concept of the microbial world (Handelsman, 2004). The implementation and advances of culture-independent phylogenetic studies demonstrated that approximately 99% of the microorganisms in certain environments could not grow in the classically used microbiological culturing methodologies (Amann *et al.*, 1995; Lefevre *et al.*, 2008). For instance, it was estimated that only 0.001–0.1 % of seawater-associated bacteria should be cultivable in defined media. This fraction increases to 0.25 % and 0.3 % for freshwater and soil bacteria, respectively (Amann *et al.*, 1995). The cultivation-independent analysis of collective microbial genomes has been generally defined as metagenomics analysis (Handelsman *et al.*, 1998; Simon and Daniel, 2009).

Cultivable bacteria have been the starting point for the discovery of many natural products with extremely important biological activities, including toxins, antibiotics, antifungals, immunosuppressants, anticancer compounds and siderophores (Banik and Brady, 2010). The application of metagenomic methodologies allowed the identification of approximately 61 distinct bacterial phyla, of which 31 have no cultivable representatives (Vartoukian *et al.*, 2010). Therefore, it is believed that the major sources of new biologically active compounds are still to be discovered from these highly diverse and uncultivable microorganisms. With this in mind, metagenomic approaches evolved from the DNA identification level to the successful heterologous expression of potential new compounds encoded in the genome of non-cultivable microorganisms. Examples include the identification of new natural products gene clusters like pederin, bryostatin and onnamide from metagenomic libraries constructed from beetles (Piel, 2002), bryozoans (Hildebrand *et al.*, 2004) and sponges (Piel *et al.*, 2004), respectively. Also, functional metagenomic studies, involving the expression-dependent screening, already

proved their usefulness (Banik and Brady, 2010). For instance, the new antibacterials turbomycin A and B were isolated from a soil metagenomic library, where *Escherichia coli* was the host strain. These small cationic molecules exhibit activity against Gram-negative and Gram-positive bacteria, including *Bacillus* species, *Staphylococcus aureus* and *Salmonella enterica* serovar Typhimurium LT2 (Gillespie *et al.*, 2002). An analogous procedure allowed the isolation and structure elucidation of the natural product palmitoylputrescine, active against *B. subtilis*. In that study, the environmental DNA (eDNA) pool from a bromeliad tank water was also heterologously expressed in *E. coli* (Brady and Clardy, 2004). All of this was possible due to the improvement of eDNA isolation, the efficient cloning of large fragments into cloning vectors and to the advanced screening methodologies (Kennedy *et al.*, 2008).

Lantibiotics and nonribosomal peptides comprise an important group of biologically active peptides. Thus, the recognition of their diversity in the environment, including the uncultured fraction of microorganisms constitutes an important field of research. Several studies focused on NRPS genes distribution and diversity has been published (Ehrenreich *et al.*, 2005; Schirmer *et al.*, 2005; Zhao *et al.*, 2008). Those studies were possible due to the existence of conserved motifs in the NRPS domains, especially those responsible for the amino acid activation (A domains), which allowed the design of degenerate primers used for their amplification (Turgay and Marahiel, 1994). To the best of our knowledge, studies focused on the diversity of lantibiotic-associated genetic determinants were not published so far.

In this chapter, the diversity of A domains from NRPS present in adverse environments such as caves and also in non-extreme environments such as lakes was investigated. With that purpose, A domains libraries were constructed based on eDNA isolated from water and soil samples of Portuguese lakes and caves, respectively. Subsequently, all the obtained domains were analyzed phylogenetically. The results obtained reveal a high diversity of A domains in these environments. Additionally, the diversity of the amino acids presumably activated by the identified domains was carried out by means of two different, yet complementary, methodologies: i) the extraction of binding pockets and comparison with Stachelhaus *et al.* (1999) and Challis *et al.* (2000) nonribosomal code and ii) the NRPS-predictor analysis described by Rausch and co-workers (2005).

7.2 Results

7.2.1 Libraries construction and sequence analysis

Herein, A domain libraries from each sampling site were constructed as described under the section 2.4.4.1 (page 57). The inserted fragment of each clone was

subsequently amplified, and subsequently subjected to restriction fragment length polymorphism analysis (RFLP) in order to distinguish between samples and thus decrease the amount of samples for nucleotide sequencing analysis. With this approach, the initial libraries of Vela lake (LV), Mira lake (LM), Barrinha de Mira lake (LB), Vale do Telhado cave (VT) and Algar Romão cave (AR) were reduced to 11, 12, 13, 16 and 14 clones, respectively. The nucleotide analysis of these clones showed that 95.5 % of the fragments showed high homology with A domains available at the GenBank database. The comparative analysis of the sequences from each sampling site revealed the presence of duplicates with dissimilar RFLP profiles. This resulted from the different orientation of insertion of each PCR fragment into the multiple cloning site of the cloning vector. Therefore, the final analysis included 7 clones from each LV and LB libraries, 5 clones from the LM library and 9 clones from each of the caves' libraries.

7.2.2 Phylogenetic analysis of A domains identified in Lakes from the central region of Portugal

All the A domains amplified from the freshwater eDNA revealed less than 79 % homology at the amino acid level with those deposited in the database. The highest scores were obtained with microorganisms belonging to the α–, γ– and δ-Proteobacteria, Cyanobacteria, Actinobacteria and Firmicutes groups. The A domains phylogenetic analysis revealed that the sampling site did not have much influence on the domains clustering (Figure 49). In fact, exactly the same or very closely related sequences were obtained from LV and LM and also from LV and LB eDNA. However, that was not observed for LB and LM samples. Approximately half of the NRPS A domains (47 %) formed monophyletic groups with species belonging to Cyanobacteria phylum, which also constituted the largest branches in the phylogenetic tree. Among them, the LV9 and LM1 domains fell on the same cluster as OciA and NdaA proteins, which are involved in the biosynthesis of cyanopeptolins and nodularins by *Planktothrix agardhii* and *Nodularia spumigena*, respectively (Moffitt and Neilan, 2004; Rounge *et al.*, 2007). The domains LV10 and LM12 were also associated with the McnC and NosC cyanobacterial NRPSs, respectively. The first participates in the production of cyanopeptolins peptides by strains of the *Microcystis* genus and the second in the production of the cyclic peptide-polyketide hybrids nostopeptolides by *Nostoc* sp. specimens (Hoffmann *et al.*, 2003; Rounge *et al.*, 2007).

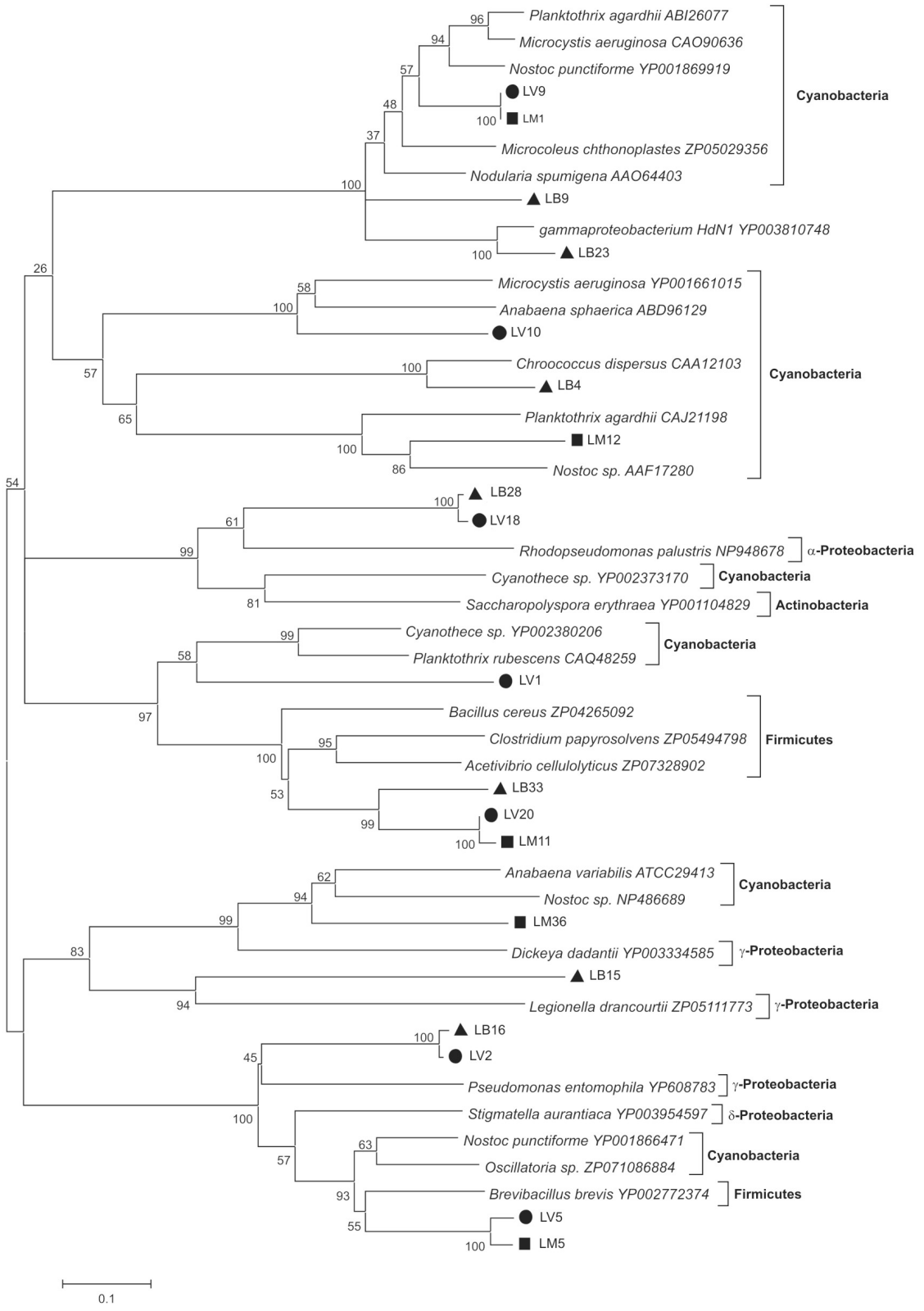

Figure 49: Phylogenetic analysis of A domains amplified from samples collected in central Portugal lakes. Sequences obtained from Lake Vela, Mira and Barrinha de Mira were evidenced with triangle, square and round labels, respectively. The bootstrap values obtained were indicated in each of the root nodes. The protein accession numbers of the closest related sequences are provided after the species name.

Here, five domains were included in the same branch as NRPS A domains from Firmicutes bacteria. LV5 and LM5 domains showed the highest homology with an GrsB catalytic residue, involved in the gramicidin production by *Bacillus brevis* (Kessler *et al.*, 2004). The other three domains (LB33, LV20 and LM11) were phylogenetically related with *B. cereus*, *Clostridium papyrosolvens* and *Acetivibrio cellulotycus* NRPSs. Identities with A domains present in bacteria belonging to the Proteobacteria phylum were also obtained. The sequences LB16 and LV2 formed a clade with an A domain of the *Pseudomonas entomophila* NRPS responsible for the biosynthesis of the pyoverdine siderophore (Matthijs *et al.*, 2009). The LB28 and LV18 domains formed a unique branch in the phylogenetic tree together with an uncharacterized NRPS present in the α–Proteobacteria *Rhodopseudomonas palustris*. The most unusual sequence was obtained from Barrinha lake sample (LB15), where the highest homology was 38% with an A domain identified in the *Legionella*-like amoebal pathogen *Legionella drancourtii* LLAP12 genome sequence (Scola *et al.*, 2004).

7.2.3 Phylogenetic analysis of A domains identified in south Portuguese caves

All the A domains amplified from soil eDNA of the two caves revealed less than 64 % homology at the amino acid level with those sequences accessible in the database. The phylogenetic analysis of these sequences revealed that samples from VT and AR caves had the tendency to cluster together, being the domains AR6, AR33 and VT8 the exception (Figure 50). The more closely related amino acid sequences were found to be from a diverse group of microorganisms belonging to the α–, β–, γ- and δ-Proteobacteria, Cyanobacteria, Acidobacteria, Verrucomicrobia and Firmicutes. Different A domains found in the present study were phylogenetically associated with NRPSs already characterized: i) the VT4 showed the highest identity with the syringopeptin-like synthetases SypB from *Pseudomonas syringae pv. syringae* and SypC from *Bradyrhizobium* sp. and ii) the AR1 and AR4 domains were clustered together with cyanopeptolins producers. However, AR1 was closer to the first subunit of that NRPSs (OciA, McnA and NcpA) and AR4 with the second subunit OciB present in *P. rubescens* genome. Other domains closely related to cyanobacterial NRPSs included AR7, AR2, AR39, VT12 and VT19. Some NRPS A domains from this environment showed homology with γ– and α-Proteobacteria such as VT20 and VT45 and VT46, respectively. A single sequence (AR37) was present in the clade containing β-Proteobacteria representatives. This included amino acid activation residues present in the *Thiomonas intermedia* K12 strain isolated from a swage pipe and in the *Variovorax paradoxus* EPS strain cultivated from soil (Jamieson *et al.*, 2009).

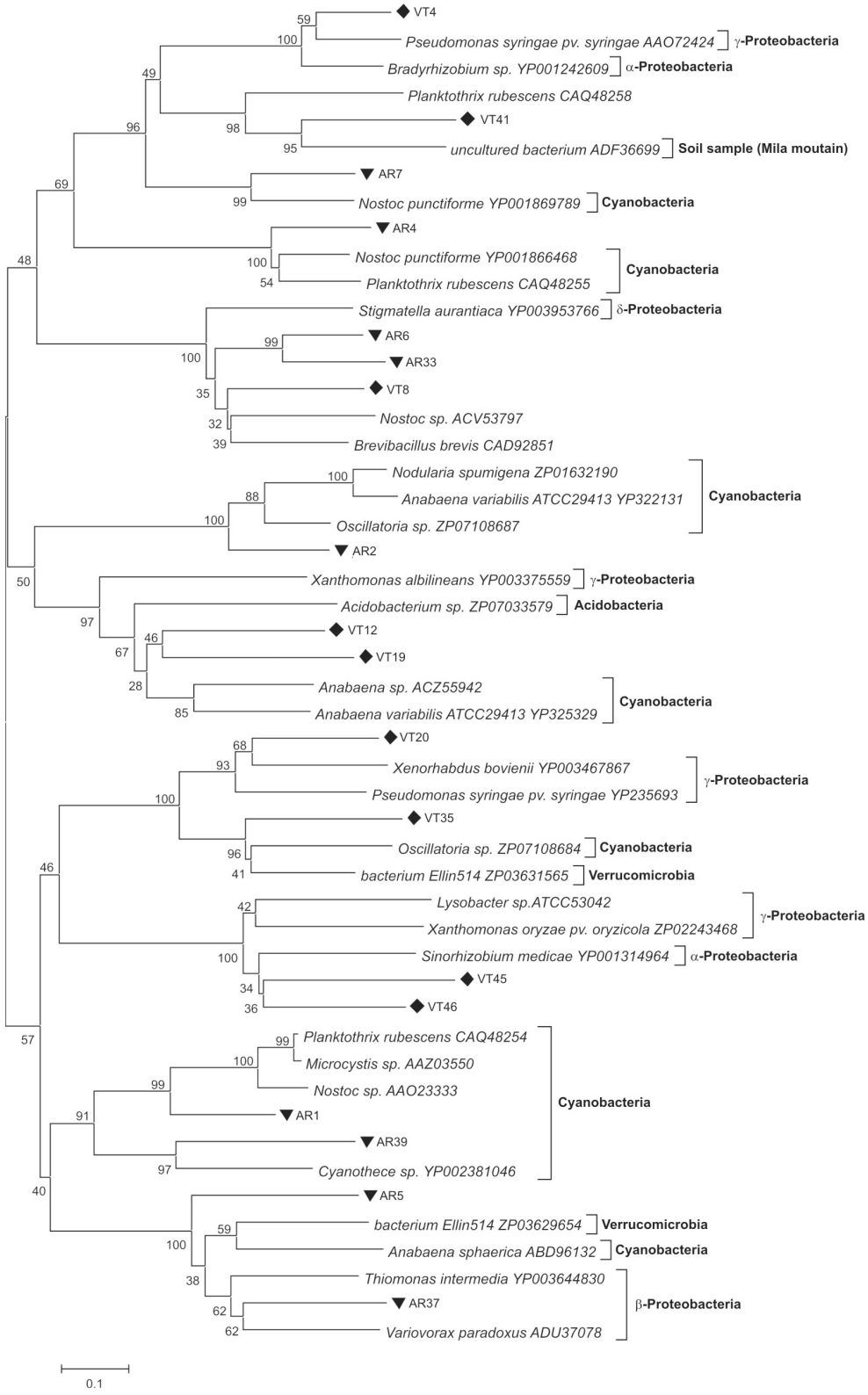

Figure 50: Phylogenetic analysis of A domains amplified from samples recovered from portuguese caves. Sequences obtained from cave Vale do Telhado and Algar Romão is marked with a losangle and an inverted triangle labels, respectively. The bootstrap values obtained were indicated in each of the nodes. The protein accession numbers of the closest related sequences were provided after the name of the specie.

The AR6, AR33 and VT8 amino acid sequences were difficult to associate to a phylogenetic group, since the closest-related matches included three distinct bacterial groups: δ-Proteobacteria, Cyanobacteria and Firmicutes. All of these A domains shared a certain degree of proximity with a domain from a bacitracin-like synthetase found in the *Stigmatella aurantiaca* genome. A similar situation was identified for the VT35 domain, which closest homologous sequences are from a Verrucomicrobia and an *Oscillatoria* sp. NRPS. A single domain amplified from the Vale Telhado cave constituted a monophylogenetic group together with an uncultured bacterium isolated from the Mila Mountain in Tibetan plateau soil (data not published).

7.2.4 Prediction of amino acid activation

The crystal structure obtained for the gramicidin synthetase A (GrsA) adenylation domain responsible for the activation of phenylalanine allowed the recognition of 10 residues crucial for the substrate binding and catalysis (Conti *et al.*, 1997; Rausch *et al.*, 2005). Based on the conserved sequence of these binding pockets, Stachelhaus *et al.* (1999) and Challis *et al.* (2000) described a specificity-conferring code (referred in the present study as Stachelhaus *et al.* code) that can be applied for the amino acid activation prediction of uncharacterized A domains. More recently, another methodology based on the physico-chemical properties of the residues lining the A domains active site was described by Rausch and co-workers (2005), which is herein mentioned as NRPSpredictor.

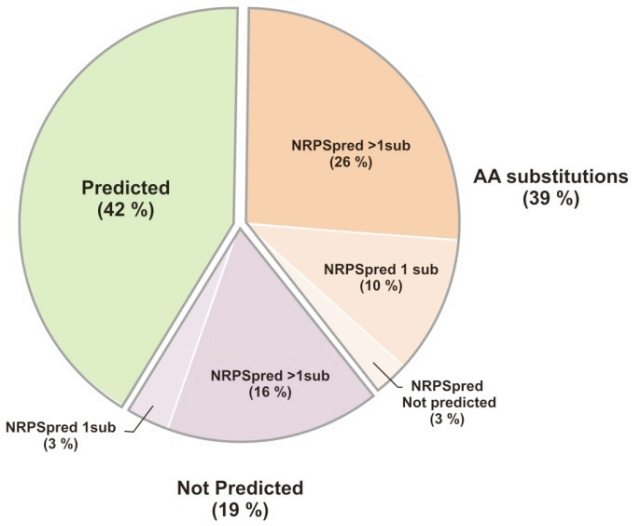

Figure 51: Results obtained for the amino acid prediction of A domains amplified in this study according to the parameters defined by Stachelhaus *et al.* code (1999; 2000). The results obtained with the NRPSpredictor are represented with the NRPSpred designation. 1sub specifies that only 1 substrate was predicted with this software, whereas >1sub that more than 1 substrate was predicted.

Here, the binding pockets of all the A domains were analyzed as described by Stachelhaus *et al.* (1999) and the resulting sequences were clustered together with those already known (Figure 51 and Figure 52). Considering the Stachelhaus *et al.* code (1999), the amino acid activation prediction was successful for approximately 42 % of the A domains obtained in the present study (Figure 51 and Figure 52) and no match was obtained for 19 % of the sequences (Figure 51 and Figure 52). Despite the occurrence of amino acid substitutions, 39 % of the sequences shared homology with the Stachelhaus *et al.* code (Figure 51 and Figure 52). Therefore, the domains belonging to these last two groups were also submitted to the NRPSpredictor analysis proposed by Rausch and co-workers (2005).

Using this approach, it was possible to identify the LV1 sequence as a probable Pro-activating domain. For the other five domains, were predictions were not possible based exclusively on the Stachelhaus *et al.* code (LB4, LV10, LB15, AR39 and AR6), equal scoring values were obtained for several amino acids. Therefore, NRPSpredictor analysis permitted the selection of a number of amino acids that are likely activated by such domains. For some domains exhibiting a few amino acid variations to the known Stachelhaus *et al.* code, NRPSpredictor approach narrowed the hypothesis of activation to a single amino acid. This was observed for the AR2 sequence, which should be responsible for the insertion of a Pro into the final nonribosomal molecule. Also, AR2 extracted binding pockets were positioned in the same clade with those responsible for Pro activation in NRPSs already characterized. A similar situation was detected for LM1, LV29 and LB33 sequences, which could be recognized as Ser-activating domains by the NRPSpredictor software. For the remaining samples, this approach extended the hypothesis to two, three or four amino acids. The clustering of the binding pockets of these sequences in the phylogenetic tree was generally coincident with one of those amino acids. An example relies on the VT35 and LV5 domains (Figure 52). According to the NRPSpredictor analysis, the highest score obtained was for Gly and Ala specificity. And, in fact, their signature sequences based on Stachelhaus *et al.* code fell into the same clade as those already described as Ala-activating domains. The prediction for the VT19 domain could not be assigned with either of the two applied approaches.

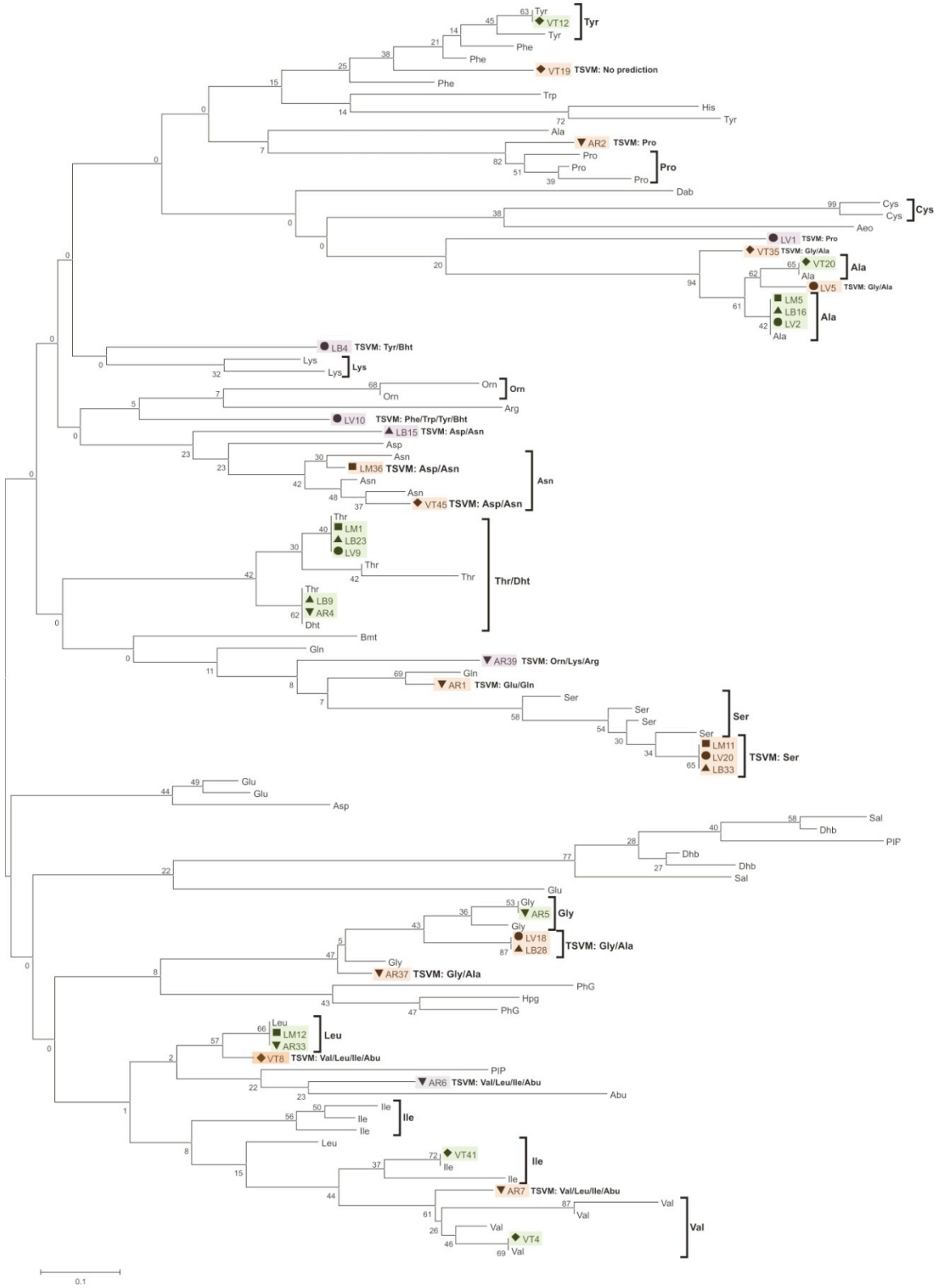

Figure 52: Phylogenetic tree with the A domain signature sequences according to Stachelhaus *et al.* (1999). Domains for which the 10 aa binding pockets were 100 % homologous to those already described were marked by green boxes. Pockets where no amino acid substitutions or no homology were observed are identified with orange and purple boxes, respectively. The results obtained for these last two groups with the NRPSprediction software were described after the TSVM designation. Abu, 3-aminopimelic acid; Bmt, (4R)-4-[(E)-2-butenyl]-4-methyl-L-threonine; Dab, 2,3-diamino butyric acid; Dhb, 2,3-dihydroxy benxoic acid; Dht, dehydrothreonine; PhG, L-phenylglycine; PIP, L-pipecolinic acid; Sal, salicylate.

7.3 Discussion

Secondary metabolites identified from microorganisms cultures have long been a major source of therapeutic agents, especially those associated with the battle against infection diseases (Zhang *et al.*, 2009). Great examples include the penicillin, vancomycin and tetracycline antibiotics. Nevertheless, programs aiming the discovery of new bioactive natural products have been (successively) declining. One of the justifications relies in the fact that microorganisms have been extensively studied and that the probabilities of finding truly novel molecules are therefore too low to be worth the effort (Singh and Pelaez, 2008). Currently two major research fields are in constant evolution to counteract such theories. One is based on introducing metagenomic approaches, that allows access to compounds encoded in the genome of uncultivable microorganisms; the other is based on the exploitation of microbial communities living within the limit of survival conditions, such as deserts, deep-sea, natural caves, mines and hot springs (Schloss and Handelsman, 2003; Reva and Tümmler, 2008; Zhao *et al.*, 2008). Since NRPS enzymes constitute a group of important drug factories from nature (Sieber and Marahiel, 2003), in the present study we investigated the diversity of A domains in two selected environments: freshwater and caves habitats.

The totality of A domains herein amplified exhibited low similarity, at the amino acid level to sequences available in protein database, demonstrating that both environments are potential reservoirs of unique and novel biosynthetic pathways. NRPS gene clusters are quite ubiquitous in the bacterial genomes of Actinobacteria, Cyanobacteria, Firmicutes, α–, β-, and δ- Proteobacteria (Donadio *et al.*, 2007). The most closely related A domains to the sequences analyzed in the present study were described for strains included in these bacterial groups and also from the Acidobacteria, δ-Proteobacteria and Verrucomicrobia phyla. The phylogenetic analysis performed revealed that approximately 42 % of the sequences obtained from eDNA from lake samples were included in the same cluster as cyanobacterial A domains. This is not surprising since the analyzed lakes represent eutrophic environments, which are widely characterized by the dominance of Cyanobacteria blooms (de Figueiredo *et al.*, 2006; de Figueiredo *et al.*, 2007). Also, the assessment of bacterial diversity from these environments by culture-independent methods revealed the predominance of specimens belonging to Cyanobacteria as well as Actinobacteria and β-Proteobacteria (de Figueiredo *et al.*, 2007). Cyanobacteria are well known producers of hepatoxic substances such as microcystins and nodularins biosynthesized by mixed PKS-NRPS (Donadio *et al.*, 2007; Tan, 2007). Therefore, a large number of nonribosomal catalytic domains from this bacterial group are presently available in the databases for comparison. Even so, no more than 78 % homology was

identified between cyanobacterial A domains and those identified in this study. Therefore, despite the possibility of an ancestral origin, these should integrate distinct multimodular enzymes that once active, can be involved in the biosynthesis of different natural compounds. Our results also revealed that some of the LV NRPS diversity detected in this study is shared with that of LM and LB. However, no similar sequences were amplified from LM and LB environments. The A domains amplified from the soil of VT and AR caves presented a lower degree of homology (64 % maximum) with NRPSs already described than those resulting from lake´s samples. In general, sequences from both sampling sites (VT and AR) did not share the same phylogenetic cluster, highlighting the differential diversity of thiotemplate modular systems that is present in such locations. Some of the most closely related sequences are described in the Acidobacteria, Verrucomicrobiota, Cianobacteria, α–, β- and γ-Proteobacteria. It is worth to mention the presence of several species associated with important biodegradative processes in nature such as nitrogen-fixation (e.g. *Bradyrhizobium* sp. and *Sinorhizobium* sp.), sulphur-oxidation (e.g. *Thiomonas* sp.) and gas reduction (e.g. *Variovorax paradoxus*). Also, some clusters integrate A domains from siderophore-producing NRPS such as *Pseudomonas syringae* pv. *syringae*. Such compounds are often involved in the uptake of iron by the microorganisms, which is essential for critical life sustainability such as DNA synthesis. In fact, caves environments are characterized by the limited amount of energy supplies mainly due to their deprivation of sunlight and energy inputs from the surface (Barton and Jurado, 2007). The comparison of the16S rDNA sequences obtained from environments around the world with the closest cultivated representatives has shown a broad diversity of bacterial groups, including members of Actinobacteria, Acidobacteria, Bacteroidetes, Firmicutes, Verrucomicrobiota, Cianobacteria, α–, β-, γ- and δ-Proteobacteria (Schabereiter-Gurtner *et al.*, 2004; Chen *et al.*, 2009; Lamprinou *et al.*, 2009). Some of them are predicted to be involved in metabolic activities that support growth in the extremely starved cave environment, such as fixing nitrogen gas from the atmosphere and energy uptake from the rock itself, providing a basis for a diverse ecosystem rather than a lifeless environment (Barton, 2006). Nevertheless, the phylogenetically distinct clades formed by VT and AR sequences indicate that the nonribosomal pathways including those amino acid activation domains have not been analyzed yet.

The A domains are responsible for the selection and activation of the amino acid that make up the final product (Finking and Marahiel, 2004). Two different methodologies were applied to predict the amino acid specificity of each sequence obtained in the present study. It was shown that the first method, described by Stachelhaus *et al.* (1999) and Challis *et al.* (2000), allowed the prediction of 42 % of the A domains. 39 % of those showed

a high homology with binding pocket sequences described in these studies, however with the presence of some amino acid substitutions. In these situations, the NRPSpredictor was used. The results obtained, which usually did stand for more than one substrate, were directly associated with the corresponding Stachelhaus *et al.* (1999) highest homologous code. Moreover, NRPSpredictor was able to generate results for the completely different binding pockets sequences extracted (19 %). Only 3 % of the A domains specificity could not be addressed by either of these two approaches. Overall, the association of the two methodologies constitute a more powerful prediction tool as previously suggested by Rausch *et al.* (2005). Also, the amino acid substitutions herein detected as well as the uncertainty of most of the predictions can result from the alternative and unknown substrate-binding patterns existent in uncultivable fractions of microorganisms.

Herein, the potential diversity of biosynthetic pathways involving NRPS in two distinct environments was demonstrated, being the caves the less explored, accessible and not influenced by anthropogenic activities. It is possible that such diversity can result in the production of novel chemical functional compounds. Thus, these locations deserve deeper studies based on metagenomic and also culture-dependent approaches to search for novel nonribosomal compounds as well as other important bioactive peptides (e.g. lantibiotics).

7.4 Experimental procedures

7.4.1 Sampling

Samples of the lakes Vela (LV), Mira (LM) and Barrinha de Mira (LB) located in central Portugal were collected as described by de Figueiredo *et al.* (2007). Soil samples were collected in sterile tubes from the Vale Telhado (VT) and Algar Romão (AR) caves, which belong to the Algarve (south Portugal) massif karstic caves. The samples were maintained in the dark at 4 ºC until the DNA extraction procedure. These samples were collected by the speologist/biologist Ana Sofia Reboleira from the LEADER lab (University of Aveiro).

7.4.2 Environmental DNA extraction

The eDNA from lake´s water samples were provided by Dr. Daniela Figueiredo (Microbial Laboratory-University of Aveiro) and extracted as described by de Figueiredo *et al.* (2007). The eDNA from soil was extracted with the UltraClean® Soil DNA Isolation Kit (MO-BIO) according to the manufacturer´s instructions. Briefly, 1 g of soil was added to the 2 mL Bead Solution tubes provided and gently mixed by vortexing. 60 μL of Solution S1

were added and the mixture inverted several times, before the addition of 200 μL of IRS Solution (Inhibitor Removal Solution). The tubes were placed horizontally on a flat-bed vortex, shaked at maximum speed for 10 min. The lysate was centrifuged at 10,000 x g for 30 sec and the supernatant was transferred to a clean 2 mL collection tube. After addition of 250 μL of Solution S2, the tubes were mixed in the vortex for 5 sec, incubated at 4 °C for 5 min and centrifuged for 1 min at 10,000 x g. The supernatant was transferred to a clean 2 mL collection tube, avoiding the pellet and 1.3 mL of Solution S3 was added. The mixture was shacked for 5 sec in the vortex and approximately 700 μL were loaded onto a Spin Filter. The solution was centrifuged at 10,000 x g for 1 min, the flow through discarded and the remaining supernatant added to the same Spin Filter and centrifuged again at 10,000 x g for 1 min. This procedure was repeated until all the supernatant has passed through the filter. The DNA was washed by the addition of 300 μL of Solution S4 of the Spin Filter and centrifuged at the same speed for 30 sec. The flow-through was discarded and the excess of Solution S4 was removed by an additional centrifugation step at 10,000 x g for 1 min. Finally, the Spin Filter was placed in a new 2 mL collection tube and the DNA was eluted by the addition of 50 μL of Solution S5 to the center of the white filter membrane and recovered by centrifugation at 10,000 x g for 30 sec. All the DNA samples were stored at - 20 °C until further use.

7.4.3 Amplification and cloning of NRPS adenylation domains

Adenylation domains were amplified from all the eDNA samples using primers MTF (5'-GCNGGYGGYGCNTAYGTNCC-3') and MTR (5'-CCNCGDATYTTNACYTG-3') (Neilan *et al.*, 1999). These degenerate primers were constructed to target the A2 and A8 core motifs, respectively. Each reaction was performed in a final volume of 25 μL using the Thermo Extensor High-Fidelity PCR master mix, with each primer at a final concentration of 1.5 pmol/μL. The amplification parameters were as follows: 94 °C for 5 min, thirty-five cycles of 94 °C for 30 sec, 51 °C for 30 sec and 68 °C for 1 min and a final extension step of 10 min at 68 °C. For each sampling site, 5 independent PCR reactions were performed, mixed together, purified and eluted in a final elution volume of 30 μl with the Jetquick PCR product purification spin kit (Genomed), according to the manufacturer's instructions. 0.5 μL of this amplification (approximately at 30-100 ng/μL) was used for re-amplification in another 5 independent reactions performed as the first PCR reactions. Samples were loaded on a 1% agarose gel and the fragment of interest (approximately 1000 bp) was excised and purified with the QIAquick Gel Extraction Kit Protocol (Quiagen), as recommended bythe manufacturer. The purified PCR product was ligated to the pCR 2.1 plasmid using the TA cloning kit (Invitrogen), according to the manufacturer's instructions.

Briefly, the ligation reaction contained 7 μL of PCR product (approximately at 50-100 ng/μL), 25 ng of the pCR 2.1 plasmid, 4 U of ligase and 1X of ligase bufferin a final volume of 10 μL and incubated overnight at 14 ºC. 5 μL of this reaction was used to transform chemically competent *E. coli* DH5α cells. Positive clones were selected based on Kan resistance and white/blue selection by incubation on LB agar plates containing 50 μg/mL of Kan and 40 μg/mL of X-Gal.

Solution:

X-Gal stock solution: dissolve 40 mg of X-Gal (Fermentas) in 1 mL of dimethylformamide. Protect the solution from the light covering the tube with aluminium foil and store at -20 ºC.

7.4.4 Restriction Fragment Length Polymorphisms (RFLP) and sequencing

For each sampling site, one hundred white colonies were randomly picked and the inserted fragment was amplified using the universal primers M13fw (5'-GTTTTCCCAGTCACGAC-3') and M13rv (5'-CAGGAAACAGCTATGAC-3'). Each reaction was performed in a final volume of 12.5 μl containing 1.5 mM $MgCl_2$, 200 μM dNTP's, 1X Green GoTaq® Buffer and 0.25 U of GoTaq® Buffer (Promega), using a bacterial colony as DNA template. The amplification parameters were as follows: 94 ºC for 5 min, thirty-five cycles of 94 ºC for 30 sec, 50 ºC for 30 sec and 72 ºC for 1 min and a final extension step of 10 min at 72 ºC. The presence of the correct amplicons (approximately 1200 bp) was evaluated on 1 % agarose gel electrophoresis. Positive PCR products were subjected to restriction fragment length polymorphism (RFLP) in order to select distinct inserts before sequencing. Each RFLP reaction was prepared with 5 μL of each of these PCR reactions (without any purification step, approximately 0.1-0.5 μg of DNA), 1X buffer *TaqI* and 10 U of *TaqI* enzyme (Fermentas), in a final volume of 20 μL. After incubation at 65 ºC for 1 hour, each digestion was loaded on a 2% agarose gel (in this step, the addition of loading buffer was not necessary). Electrophoresis was performed at 120 V during 1 hour.

The pattern of the fragments digestion was analyzed with the GelCompar II software (Applied Maths) and only one representative of each clustered group was submitted to nucleotide sequencing reaction and further nucleotide sequence analysis (STABVIDA-Portugal).

7.4.5 Phylogenetic analysis

All the sequenced A domains nucleotide sequences were first analysed using BlastX program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The two sequences exhibiting the highest

homology with the A domains obtained in this study were selected. Sequentially, all the deduced amino acid sequences between two conserved core motifs (A4 and A5) were aligned using Clustal X. This alignment was the basis for the phylogenetic analysis performed withMEGA 4.1 software (Tamura *et al.*, 2007), using as parameters the Poison correction model for amino acids and a bootstrap consisting of 1000 replications.

The amino acids constituting the binding pockets of each sequence were extracted as described previously by Stachelhaus *et al.* (1999) and aligned with some of those already known. The alignment of these 10 amino acids was performed with the following parameters: gap penalty 12 and gap length penalty 6. The corresponding phylogenetic tree was constructed as abovementioned. The prediction of selectivity-conferring code of A domains by the NRPSpredictor approach (Rausch *et al.*, 2005) was performed on the web-based platform http://www-ab.informatik.uni-tuebingen.de/software/NRPSpredictor.

CHAPTER VIII

Synopsis and future perspectives

8.1 Production of antagonistic compounds by *Bacillus licheniformis* I89

Bacillus licheniformis has been used for decades in the manufacture of industrial enzymes including proteases and α-amylase (Rey *et al.*, 2004). Its success in certain industries is mainly due to the their ability to produce and secrete large quantities (20-25 g/L) of such compounds (Schallmey *et al.*, 2004). For instance, specific *B. licheniformis* strains are used to produce industrially the nonribosomal antibiotic bacitracin (Waschkau *et al.*, 2008). The *B. licheniformis* I89 strain was isolated from a hot spring environment in São Miguel (Azores Island). Thus, this bacterium was routinely grown at 50 ºC (Mendo *et al.*, 2000). Extracts of I89 strain culture supernatants were able to inhibit the growth of the closely related *Bacillus subtilis* and other Gram-positive bacteria such as *Micrococcus luteus* and *Staphylococcus aureus*. It was also shown that the activity of the compound produced by *B. licheniformis* I89, and responsible for the inhibition observed in the above referred strains, was not affected by some proteases, including α-chymotrypsin and trypsin (Mendo *et al.*, 2004). In the same study it was determined that a peptide with M= 3249.7 Da was contributing for the biological activity. The compound was designated as Antibiotic 89 (A89). *B. licheniformis* strains are known to produce several bacteriocins (chapter II). However, only for *B. licheniformis* ZJU12 an antibacterial compound within the molecular mass range of A89 peptide was later identified. Nevertheless, in the ZJU12 strain, antibacterial activity was completely abolished when the active compound was incubated with trypsin, whereas A89 was not affected by this protease, suggesting the distinctive nature of the active compounds (He *et al.*, 2006). Considering these facts, it was recognized that the I89 strain was a producer of, at least, a novel antibacterial compound. Thus an investigation was started in order to clarify the biological origin of the peptide as well as to get some insights on its chemical structure.

In the present study, the antibacterial activity of the compounds produced by *B. licheniformis* I89 was higher when cells were grown at 37 ºC and for that reason all the experiments and results that are reported in the present dissertation were obtained at this temperature. Moreover in this study it was shown that the mixture of antibacterial compounds produced by I89 strain (where A89 is included) is active against the Gram-positive bacteria *Enterococcus faecalis*, *Enterococcus faecium*, *Haemophilus influenzae*, *Listeria monocytogenes* and *Staphylococcus aureus*, including a clinical methicillin-resistant strain (MRSA), highlighting the interest of this bacterial strain and its bioactive compounds (chapter II). Thus, one of the aims of the present study was to separate the antibacterial components present in *B. licheniformis* I89 supernatants and obtain enough concentration of pure A89 peptide to proceed with further characterization procedures. To accomplish this objective, a protocol including solid phase extraction followed by

preparative HPLC and bioassay was developed. During this procedure, other compounds with the ability to inhibit *M. luteus*, the indicator strain of this study, were detected. Among these compounds, a peptide characterized by the molecular mass of 3020 Da, that was designated as B89 and selected for further characterization, similarly to A89 (chapter II). These results were not surprising, since it has been reported that some strains of the *B. subtilis*-group, where *B. licheniformis* is included, can in fact produce more than one antibacterial compound (Stein, 2005). To get some information of the amino acid composition of these two peptides, Edman degradation on the pure compounds was attempted, however with no success. The blockage of Edman degradation reactions has been associated with compounds harboring unsaturated amino acids such as Dha or Dhb (Holtsmark *et al.*, 2006). Therefore, it was considered that A89 and B89 could possess such residues on their structure, similarly to lantibiotics. Surprisingly, during the separation process, it was observed that the bactericidal activity of A89 and B89 pure peptides against the indicator strain was decreased compared to the situation when both peptides were applied together, suggesting that they could act synergistically (chapter II).

Regarding the biological nature of these compounds, it was found that the NRPSs present in the *B. licheniformis* I89 strain (lichenysin and putative siderophore) have high homology with those identified in the genome of *B. licheniformis* ATCC 14580 and *B. licheniformis* DSM13 (isogenic strains) (Rey *et al.*, 2004; Veith *et al.*, 2004). It was verified that the putative products of those enzymes do not correspond to the A89 and B89 peptides (chapter II). However, the analysis of both of the isogenic strains genome revealed the presence of a putative two-component lantibiotic gene cluster, which product was designated as lichenicidin (Rey *et al.*, 2004). The transcription of the *licA1* structural gene present in this cluster, followed by modification of the corresponding LicA1 prepropeptide implying the occurrence of 7 dehydration reactions followed by the removal of the leader sequence (after the GG-motif) would result in the production of a peptide with approximately M= 3250 Da. The same principle applied to the identified *licA2* gene, but considering the occurrence of 12 dehydration reactions and the removal of the -NDVPE-sequence after the LicA2 leader sequence, would originate a lantibiotic with M= 3020 Da. Therefore, considering this information, the molecular masses of the lichenicidin gene cluster products were coincident with those observed for the A89 and B89 peptides. After PCR amplification and nucleotide sequencing it was confirmed that *B. licheniformis* I89 strain also harbored the lichenicidin structural genes. Thus, it was considered that A89 and B89 peptides corresponded to the lichenicidin two-component lantibiotic. Concurrently, Begley and coworkers (2009) also suggested that these peptides produced by *B. licheniformis* ATCC 14580 corresponded to the lichenicidin lantibiotics and

designated them as Bliα (M= 3250 Da) and Bliβ (M= 3020 Da). Their study established that separately, each of the peptides possessed antimicrobial activity. However, synergism was apparent when their individual fractions were combined, similarly to that already observed in the present study. Later on, Dischinger and co-workers (2009) were actually able to associate the *B. licheniformis* DSM13 lichenicidin gene cluster with the production of both peptides, inducing the abolishment of their production by the insertion of a resistance cassette in the *licA1* and *licA2* structural genes. Surprisingly, in these two strains the peptides could not be detected in the bacterial supernatant and were considered to be cell wall associated. The limited diffusion of lichenicidin to the supernatant was explained by the fact that *B. licheniformis* is able to produce the glutamyl polypeptide capsule which could trap the positively charged Bliα (+1) and Bliβ (+2) peptides (Dischinger *et al.*, 2009). However, in I89 fermentations both compounds were always detected and purified from the bacterial supernatants, despite their identification also in cell wall extracts. Thus, either the I89 strain's capsule has slight differences from that of these two type strains, or the production media used promoted the diffusion of the peptides. Therefore, it would be interesting to investigate if the Bliα and Bliβ peptides are released to the culture supernatant of ATCC 14580 and DSM 13 strains when grown in medium M. Similar to what happens with the I89 strain, the DSM 13 strain produces other antibacterial compounds, apart from lichenicidin, which were not characterized so far. For instance, *Staphylococcus gallinarum* Tü 3928 (producer of the gallidermin lantibiotic) and several *Staphylococcus aureus* strains (including MRSA) were exclusively inhibited by DSM 13 cell wall associated extracts, whereas *Enterococcus faecium* strains were only affected by the antibacterial compounds present on its culture supernatant. Yet, it is worth to mention that both extracts were active against *M. luteus* strains. Therefore, it could be hypothesized that probably some of the antibacterial compounds detected in the DSM 13 supernatant can also be produced by the *B. licheniformis* I89 strain. Some of these compounds might be present in the fractions that were identified in the present study during the separation of the *B. licheniformis* I89 supernatants but that were no further investigated (chapter II). Regarding the biological nature of these unidentified compounds, it would be expected that they will be part of the group of non-modified bacteriocins, since in the *B. licheniformis* DSM 13 genome, apart from lichenysin and one putative siderophore nonribosomal cluster, no polyketide synthetases or other lantibiotic gene clusters were identified. Therefore, the investigation of the biological and structural origin of these other compounds would be interesting to perform in future studies.

Meanwhile, along the routine cultivation of *B. licheniformis* I89 strain on agar plates, two different colonies phenotypes were observed: the parental and rough colonies and new

simple and smooth colonies (chapter III). The factor that triggered such phenomenon could not identified so far. Nevertheless, it was found that the smooth phenotype was quite stable, since its subsequent cultivation did not result in its reversion to the rough phenotype, suggesting that such variation was not due to the bacterial bistability. For *B. subtilis* it was observed that *spo0A* (the master regulator for the beginning of the sporulation process) mutants develop flat colonies consisting on non-adherent cells (Branda *et al.*, 2001; Hamon and Lazazzera, 2001). However, for the I89 strain it was not possible to correlate the morphological alteration with a difference in the sporulation rate. Additionally, the switch of a bacterial phenotype is normally accompanied by genetic alterations such as mutations, insertions or deletions. These random rearrangements can occasionally cause the improvement as well as the damage of some interesting characteristics of the parental strain, such as the abolishment of secondary metabolites production (Berditsch *et al.*, 2007; Brosch *et al.*, 2007). However, generally, the antagonistic activity of both phenotypes did not present major discrepancies. Nevertheless, that activity was found to be a consequence of a mixture of antibacterial compounds. Since the main focus of this thesis lies on the characterization of the Bliα and Bliβ peptides, it was important to understand whether the rough to smooth transition affected their production rate. The results revealed that a reduced abundance of the Bliβ peptide (and its derivative) was associated with the smooth colonies, even in medium M the medium selected for the peptide's production. Yet, the reasons behind this difference were not the objective of the present study, and were not investigated. However, they can be part of the *B. licheniformis* I89 "domestication" process. Thus, it should be interesting in the future to investigate which processes were altered in the smooth variant that had a negative impact in the lichenicidin production. For this, the analysis of protein expression patterns of both phenotypes can help to clarify this aspect, since it was observed that both phenotypes could indeed present differences at this level. Alternatively, the transcriptome of both variants could be characterized by the new generation of sequencing technologies. The retrieved information would provide relevant data on which future research could be built up.

So far, the main application of lantibiotics lies in food technology (Gálvez *et al.*, 2007). Nisin was approved as biopreservative in Europe and the US and is included into a broad range of products (e.g. dairy products, liquid egg, bakery products, vegetables, meat and fish) to prevent microbial spoilage and inhibit the outgrowth of clostridial spores (Delves-Broughton *et al.*, 1996; Lubelski *et al.*, 2008). Clinically, lantibiotics have been proposed for the treatment of infections caused by antibiotic resistant bacteria. For instance, the application of the class II lantibiotic mersacidin to a mouse rhinitis model was able to

eradicate MRSA (Kruszewska *et al.*, 2004). In fact, the medical application of this lantibiotic is already in development (pre-clinical stage) in Novacta Biosystems Lda. (Hatfield, England) (Hancock and Sahl, 2006). The related lantibiotic actagardine showed a great activity against streptococci and the MIC data obtained for microbisporicin seem quite promising against MRSA and enterococci (Castiglione *et al.*, 2008; Bierbaum and Sahl, 2009). Other advantage of lantibiotics resides in its reduced susceptibility to the action of proteases (mainly due to the presence of the thioether rings) and therefore not prone to easy degradation or antibody formation (Bierbaum and Sahl, 2009). The emergence of antibiotic resistance and the quicker ineffectiveness of new generation conventional antibiotics are alarming to medical practitioners. This has been one of the major concerns since the beginning of antibacterial therapy, with the clinically introduction of penicillin in 1944. At this time, approximately 6 % of the *Staphylococcus aureus* isolates were already resistant to the antibiotic and six years later this number increased to approximately 50 % (Livermore, 2000). Concerning the two-peptide lantibiotics, it has been highlighted that they have a broad range of activity and that the development of resistance is rare (in the case of lacticin 3147) (Bierbaum and Sahl, 2009). In this context, it would be appealing to carry out experiments with the lichenicidin peptides and involving a larger number of Gram-positive bacterial strains from each of the species that are inhibited by these peptides to obtain appropriate minimal inhibitory concentration (MIC) values. Also, the lichenicidin hemolytic and cytotoxic potential should be evaluated. Moreover, the development of resistance to the lichenicidin lantibiotic could be estimated by the continued exposure of bacterial cells to this lantibiotic.

8.2 Biosynthesis of lichenicidin in *Escherichia coli*

The construction of a *B. licheniformis* I89 genomic library, revealed the presence of some clones, containing the lichenicidin gene cluster, that were able to inhibit the growth of *M. luteus*. After the confirmation that these clones were producing both Bliα and Bliβ peptides, a clone designated by *E. coli* Lic5 was selected as a lichenicidin heterologous expression system. This was very surprising and overthrown the dogma of 20 years of research on lantibiotics: the production of bioactive lantibiotics by *E. coli* was not possible (chapter IV). In the last years, semi-*in vitro* biosynthesis (SIVB) of nukacin ISK-1 and bovicin HJ50 lantibiotics in *E. coli* were described (Nagao *et al.*, 2007; Lin *et al.*, 2010). Such systems combined the *in vivo* modification of the prepropeptide by the co-expression of the structural gene and the modifying enzyme followed by the *in vitro* cleavage of its leader peptide. However, in the present study, Bliα and Bliβ production was completely achieved

in vivo in the Gram-negative host. The application of the SIVB methodology permits the recovery of potentially toxic peptides avoiding at the same time the regulatory processes involved in the biosynthesis of several lantibiotic. On the other hand, it does not allow the investigation of the pathways involved in their production (e.g. export, regulation and self-protection of the producer strain). And, for instance, the characterization of lichenicidin biosynthesis process was one of the major objectives of the present study. In this context, the generation of knockout mutants for the lichenicidin gene cluster in the original producer *B. licheniformis* I89 would have been a difficult method due to its low transformation efficiencies. Actually, it has been mentioned that transformation of *B. licheniformis* strains routinely requires long periods of time and great experimental efforts to finally obtain a desired transformant (Waschkau *et al.*, 2008). Accordingly, the successful production of lichenicidin by *E. coli* opened up great perspectives for such proposes, involving less time-consuming procedures. Thus, all the genes constituting the lichenicidin biosynthetic cluster were deleted and the impact of these modifications in the production of Bliα and Bliβ was evaluated. With this approach, it was possible to establish that in the absence of LicA1, LicM1, LicT and LicR proteins, the synthesis of Bliα does not occur. Regarding Bliβ, its production was always abolished if LicA2, LicM2, LicT, LicP and LicY were deleted from the system. Finally, both peptides were synthesized without the transcription of the LicX and the LicFGEHI elements (Chapter IV). These results, together with the considerable amount of information available for the lantibiotics biosynthesis revealed that the production of Bliα and Bliβ peptides involves the following essential steps: i) the production of LicA1 and LicA2 prepropeptides by the expression and translation of *licA1* and *licA2* genes, respectively, ii) the LicM1 modifying enzyme dehydrates 7 Ser/Thr residues of the LicA1 propeptide and catalyzes the formation of thioether rings, whereas LicM2 would carry out the same reactions, however 12 dehydrations of Ser/Thr amino acids will be achieved on the LicA2 propeptide, iii) both modified prepropeptides would be exported and their leader peptides removed at the double Gly motif by LicT, producing the biological active Bliα peptide and the inactive Bliβ' and iv) the LicP protease would process the trimming of the 6 N-terminally located amino acids of Bliβ' to produce the biologically active Bliβ peptide (Figure 53). The precise role of LicR and LicY in this biological process was not investigated in the present study. However, LicR, which possesses a helix-turn-helix domain, should constitute a single component-signaling regulator. The fact that in the absence of LicR, the Bliα peptide was not produced indicates that this protein should promote the expression of the *licA1*-containing operon, rather than repress it as observed for the lacticin 3147 single regulator LtnR. In several class I lantibiotic systems such as nisin and subtilin it has been shown that the signal triggering the

LanR functionality is the presence of the lantibiotics in the bacterial supernatant. In the present work, the potential of Bliα auto-regulating its own synthesis was not evaluated. The influence of LicY on the Bliβ production is more difficult to predict because its counterparts are not commonly found in the lantibiotic gene clusters. This small protein harbors three transmembrane helices, which suggests its location in the membrane. Its involvement in the regulation of Bliβ biosynthesis is not considered, since no domains or homology with putative regulators were detected for LicY. In fact, LicY possesses a degree of similarity with an uncharacterized protein of *B. halodurans* genome and with a C-terminal processing protease from the Gram-negative *Borrelia burgdorferi*. Therefore, it was considered that LicY could be involved in the maturation of the LicP protease, which is also only involved in the production of the Bliβ peptide. However, this hypothesis was not confirmed since an active LicP was obtained by expression of the *licP* gene alone, and thus in the absence of LicY protein (chapter IV). Consequently, the role of this protein in the Bliβ biosynthesis would deserve to be studied in more detail.

Regarding the putative immunity LicFGEHI elements, it was observed that their inactivation was not accompanied by an increase of the lichenicidin susceptibility in the *E. coli* producer strain. This was understandable, since the *E. coli* outer membrane forms a natural barrier for Bliα and Bliβ permeabilization. And, if these peptides are not able to reach the bacterial cytoplasm, their export back to the supernatant through the active ABC-transporter LicFEG will not be necessary. However, a decrease in the abundance of the Bliα and Bliβ peptides was identified when *licFGEHI* elements were not expressed (chapter V). It would be interesting to establish the origin of these differences in upcoming experiments.

The most surprising feature of the lichenicidin production by a Gram-negative host was the presence of the peptides in the bacterial supernatant. The export systems of Gram-negative bacteria have an additional impediment in the cell wall to overcome – the presence of an outer membrane (OM). Thus, in the beginning it was not clear if the presence of Bliα and Bliβ peptides in the *E. coli* Lic5 supernatant resulted from real transport or if it was due to bacterial lysis. The lantibiotics prepropeptides, including LicA1 and LicA2 lack a typical N-terminal for the general *sec* secretory pathway. In Gram-negative bacteria, the proteins that have this property are usually secreted directly from the cytoplasm to the extracellular environment through a type-I *sec*-independent dedicated system. An example of that is the secretion of colicin V and hemolysin, which are non-modified bacteriocins. For instance, the secretion of colicin V by *E. coli* involves the complex CvaB-CvaA-TolC in which CvaA and CvaB are encoded in the colicin V gene cluster and TolC is an *E. coli* outer membrane protein (Gilson *et al.*, 1990; Skvirsky *et al.*,

1995). The CvaB protein is characterized by six transmembrane domains with a typical ATP-binding cassette on its C-terminus, whereas CvaA is a membrane fusion protein (MFP) comprising a N-terminal hydrophobic domain presumably responsible for its attachment to the inner membrane (Gilson *et al.*, 1990; Skvirsky *et al.*, 1995). Hwang *et al.* (1997) showed that CvaA directly interacts with CvaB and TolC, thus allowing the transport of ColV to the bacterial supernatant without any periplasmic intermediate sub-location. The potential involvement of the TolC OM protein in the Bliα and Bliβ export was also evaluated by expressing the lichenicidin biosynthetic cluster in cells without this gene. In such conditions, none of the lantibiotics were detected in the culture supernatant. Consequently it was confirmed that Bliα and Bliβ are actually transported through the OM, with the involvement of the TolC protein and their presence on the bacterial supernatant is not due to bacterial lysis. For this process, the ABC-transporter/protease LicT most probably will interact with the TolC protein to achieve the translocation of Bliα and Bliβ across the *E. coli* outer membrane (Figure 53). Whether this association requires or not a MFP, provided by the *E. coli* host, is a question that should be further explored. Alternatively, it can be considered that the LicT protein is anchored into the cytoplasmatic membrane by its transmembrane domains, being its ABC transporter C-terminus facing the cytoplasm and its N-terminus protease directed to the periplasmic space, where it can interact with TolC. The haloduracin transporter HalT also includes an N-terminal protease motif, 6 transmembrane motifs and a C-terminal ABC-cassette, similarly to LicT. In fact, in chapter VI of the present dissertation, it was demonstrated that HalT is able to substitute the LicT function on the export of the lichenicidin peptides. Thus, it is actually possible that their structural features are able to directly interact with the TolC protein. The elucidation of the precise mechanism of lichenicidin secretion in *E. coli* is therefore a key aspect that will help the understanding of the entire biosynthetic process in this Gram-negative host.

In chapter V, an *E. coli* strain with a permeable OM was shown to be susceptible to lichenicidin. Also, an *E. coli* Lic5 strain was still able to secrete both peptides in the absence of the putative immunity genes *licFGEHI*. In this mutant, if Bliα and Bliβ were transported via the periplasm, the bacteria would most probably not survive. These results also supported the hypothesis of the periplasm-independent transport of both Bliα and Bliβ peptides, unless they coexist in an inactive complex composed by Bliα and the untrimmed Bliβ peptide (Bliβ'). The precise role of each of the *licFGEHI* genes in the lichenicidin self-protection could not be evaluated in the course of this study. Nevertheless, it will be performed in future experiments using the lichenicidin susceptible *E. coli imp*4213 strain. This strain will be transformed with several plasmids harboring distinct combinations of

each of the *licFGEHI* genes, followed by the evaluation of the increase or decrease of susceptibility to lichenicidin of each of the constructed strains.

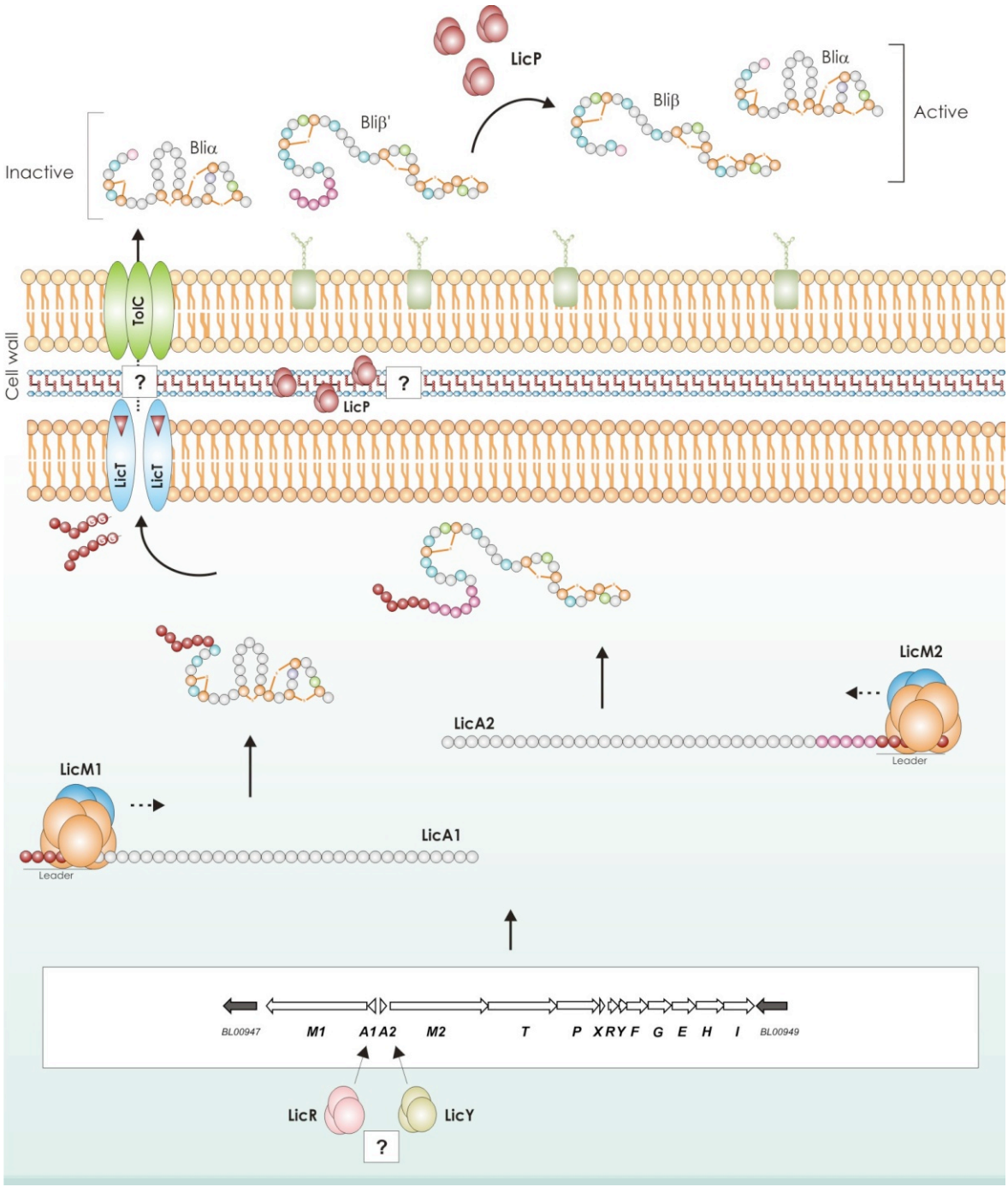

Figure 53: Representation of the general biosynthetic pathway of lichenicidin Bliα and Bliβ peptides in the Gram-negative *E. coli* Lic5 strain. The question marks represent the interactions, which were not completely elucidated in the course of this thesis.

8.3 Bioengineering of lantibiotics in *Escherichia coli*

Due to its ribosomal origin, the construction of lantibiotic variants with potentially altered biological and chemical properties can be performed in a more direct fashion when compared to that of conventional antibiotics (Field *et al.*, 2010). Therefore, a *trans* complementation system able to easily produce lichenicidin variants in *E. coli* was established in the present study (chapter IV). Initially, this approach was employed with the objective of getting some insights on the Bliα and Bliβ structures. To that end, all the Ser, Thr and Cys residues in the lichenicidin peptides were substituted by Ala residues using site-directed mutagenesis of their structural genes. The molecular masses of the produced lichenicidin variants allowed the revision of the position of the dehydrated residues in the Bliβ peptide previously proposed by Begley *et al.* (2009) (Figure 54). Moreover the difference of + 1Da between the Bliα and Bliβ molecular masses obtained by high-resolution mass spectrometry and those previously calculated suggested the presence of a 2-oxobutyril residue in the N-terminus of both peptides. Moreover, the MS/MS analysis of the wild-type peptides produced by *B. licheniformis* I89 and *E. coli* Lic5 in conjugation with the MS/MS analysis of a Bliβ variant, allowed the establishment of the pattern of thioether rings for Bliα and Bliβ that differed from that previously anticipated by Begley *et al.* (2009). Recently, the structures of Bliα and Bliβ peptides (designated by Lchα and Lchβ) produced by *B. licheniformis* VK21 strain were elucidated by NMR spectroscopy (Shenkarev *et al.*, 2010). The results obtained by these authors were generally in agreement with the structures determined in the present study, differing exclusively in the residues involved in the formation of the Bliα A-ring (Figure 54).

Some of the mutated peptides were barely or even not produced. This was particularly obvious when changes were performed in the residues involved in the formation of the C-terminal thioether rings. The reasons for such results can involve the incompatibility of these peptides with the native biosynthetic, transport, regulation or immunity machinery. To overcome these uncertainties, the development of a SIVB system for the lichenicidin production could be an appealing approach for future work. With such system, it would be more likely that all the mutated peptides will be recovered for bioactivity assays. In fact, the production of lantibiotic derivatives with enhanced and/or a wider spectrum of activity is one of the major goals of lantibiotics bioengineering. Nowadays, the identification of lantibiotics variants with enhanced activity is becoming more frequent, however this is only possible due to the creation of larger banks of engineered peptides (Field *et al.*, 2010). For the lichenicidin peptides, these libraries are still not available and will constitute the aim of future studies. Moreover, the assays of antagonistic activity should be

enlarged to other strains besides *M. luteus*, including Gram-negative representatives such as *E. coli* and *Pseudomonas aeruginosa*.

Regarding the lichenicidin mode of action, it was recently proved that similarly to lacticin 3147 and haloduracin, the two peptides can act synergistically at a molecular ratio of 1:1 to exert their maximal antibacterial activity (Shenkarev *et al.*, 2010). Separately, Bliα and Bliβ have biological activity, but in these circumstances, higher concentrations of each peptide are required to obtain bacterial inhibition. These findings were quite surprising, since the Bliα peptide is similar to the mersacidin lantibiotic. The conserved Glu residue among the mersacidin-like peptides was predicted to play an essential role in lipid II-binding. Indeed, the substitution of BliαGlu26 by Ala resulted in abolishment of antibacterial activity (chapter IV). However, the minimal inhibitory concentration (MIC) of Bliα against *M. luteus* is above to 15 μg/mL, whereas for mersacidin is about 13 times lower (1.2 μg/mL) (Appleyard *et al.*, 2009; Shenkarev *et al.*, 2010). Therefore, some differences on their mode of action can be predicted, suggesting that the interaction between the Bliα peptide and the lipid II molecule is distinct from that of mersacidin and should therefore be further investigated.

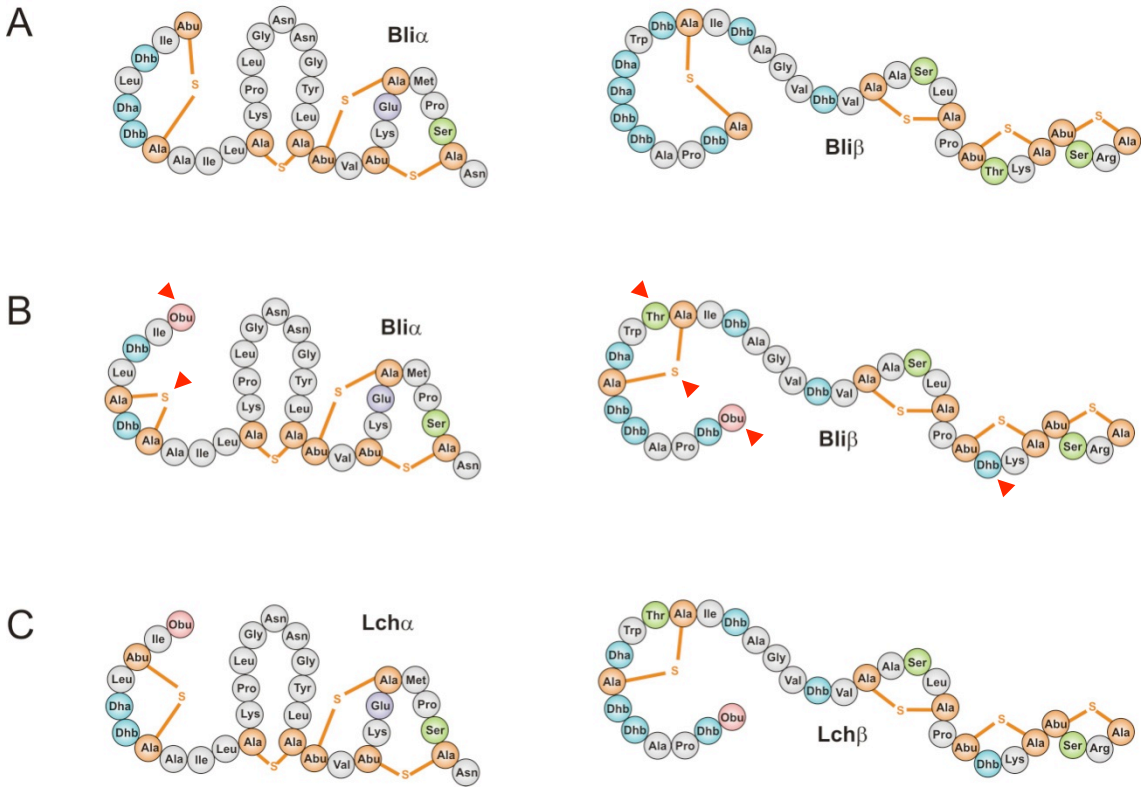

Figure 54: Representation of the different structures of lichenicidin peptides. A corresponds to the structures proposed by Begley and co-workers (2009), B corresponds to the structures proposed in the present study (Caetano et al., 2011) and B the NMR elucidated structures by Shenkarev et al. (2010). The differences are highlighted with red triangles on Bliβ peptide.

The heterologous systems, as those developed during the present study, are quite amenable to genetic manipulation and can therefore be potentially useful for the production of: i) compounds which have never been characterized but which gene clusters are known or ii) compounds that are produced in very low amounts by its original producers. With this in mind, the haloduracin production using the lichenicidin biosynthetic machinery was attempted using chimeras of HalA1 and HalA2 propeptides N-terminally fused with the LicA1 and LicA2 leader sequences, respectively (chapter VI). Results showed that the biosynthetic and/or regulatory machinery of lichenicidin was not malleable enough to produce biologically active Halα and Halβ peptides. Nevertheless, HalT was able to export and process the leader sequence of LicA1 and LicA2 fully modified peptides. These findings highlighted its substrate flexibility and also showed that Lic-leader sequences are not necessary for its functionality. Therefore, it is possible that the co-expression of a class II prepropeptide with the corresponding LanM and LanT (or alternatively LicT) proteins in *E. coli* can be more successful in the production of other fully active lantibiotics than the expression of chimeras in the presence of all the biosynthetic elements.

8.4 Metagenomics and new antibacterials

The phylogenetic analysis of the genetic elements designated as adenylation (A) domains involved in the biosynthetic pathway of conventional antibiotics was performed. For that, a cultivable-independent approach was employed to samples collected from different lagoons in the Central region of Portugal and also from more unusual environments such as caves. The applied methodology is relatively simple to perform and exclusively allows the recognition of potential new nonribosomal peptide synthetases, which can possibly produce new compounds, including new antimicrobials. A large diversity of A domains was retrieved from both environments. The majority of these sequences showed low similarities with sequences already deposited in the databases, highlighting the presence of potential novel nonribosomal peptide synthetases in these environments. The construction of metagenomic libraries, will allow the access to some of the compounds produced by such enzymes. In fact, some industries, including pharmaceuticals, are interested in exploiting the resources behind the uncultivable fraction of microorganisms present in several distinct environments (Lorenz and Eck, 2005). Additionally, the screening of such libraries based on the clones' antibacterial bioactivity rather than on their genetic content will not only allow the identification of potential new

nonribosomal antibiotics but also and maybe more frequently of novel bacteriocins, including lantibiotics.

8.5 Highlights and major conclusions of the study

The present study contributed greatly for the characterization of some of the antibacterial compounds produced by *B. licheniformis* I89. Additionally, allowed new advances in the investigation of the biosynthetic machinery of some of these compounds. More specifically:

- *B. licheniformis* was identified as a lantibiotic producer, which was not recognized before the beginning of this study. Herein, it was proved that the I89 strain produces the two-component lantibiotic peptide lichenicidin (Bliα and Bliβ).

- The production of lichenicidin seems to be affected by the phenotypic dissociation of *B. licheniformis* I89 when it occurs.

- For the first time, a lantibiotic was fully produced totally *in vivo* in the Gram-negative host *E. coli*.

- It was established that *licA1*, *licM1*, *licT*, *licR* are involved exclusively in the Bliα biosynthesis, whereas *licA2*, *licM2*, *licT*, *licP* and *licY* are directly responsible/related with the Bliβ synthesis. The putative immunity genes *licFGEHI* and the *licX* are not involved in the lichenicidin production.

- New components of two-peptide lantibiotics were identified: LicP and LicY.

- It was established that the *E. coli* host is able to export Bliα and Bliβ through the outer membrane mediated by the TolC outer membrane protein.

- The *trans* complementation system developed is able to produce new variants of the Bliα and Bliβ peptides was developed.

- The structural features of the Bliα and Bliβ peptides were revised.

- The biosynthetic machinery of lichenicidin did not show enough flexibility to produce the closely related lantibiotic haloduracin in *E. coli*, when directed by the lichenicidin leader sequences.

Moreover, it was also established that the communities of some Portuguese lagoons and caves contain a high diversity of nonribosomal adenylation domains, which indirectly showed that these environments enclose new peptide synthetases, which can produce potentially novel compounds with interesting activities, such as antimicrobial peptides.

8.6 Future perspectives

It is believed that this study raised more questions than answers. Therefore, the information retrieved, together with the implementation of new protocols should provide the basis for future studies, especially those regarding the biosynthesis and bioengineering of lantibiotics.

The major points requiring further investigation were raised along this final chapter, and and are here summarized:

- The characterization of the other *B. licheniformis* antibacterial compounds should be performed.

- The yields of lichenicidin production by *B. licheniformis* I89 and *E. coli* Lic5/BLic5 should be estimated and compared.

- The contribution of LicR and LicY to the biosynthesis of Bliα and Bliβ peptides should be elucidated as well as the contribution of each LicFGEHI element to the lichenicidin self-protection mechanism. At this stage it will also be possible and appealing to perform cross-immunity studies with other class II lantibiotics.

- The sub-cellular location of LicP in *E. coli* as well as its proteolytic specificity should be unveiled.

- The interaction of LanT and TolC needs to be clarified in order to the completely understand the export of lantibiotics by *E. coli*.

- The contribution of LicFGEHI for the lichenicidin self-protection mechanism should be investigated.

- The importance of the Bliα and Bliβ structure to their bioactivity should be further investigated by the production of new lichenicidin variants.

- The production and purification of lichenicidin variants for bioactivity testing can benefit from the establishment of a SIVB system for lichenicidin accompanied by the introduction of a C-terminal six-histidine tag to Bliα and Bliβ peptides.

- The extraction and/or mass spectrometry methodologies should be optimized to allow the identification of some lichenicidin variants, for instance those possessing additional amino acids.

- The production of other lantibiotics in *E. coli* totally *in vivo* (with or without the expression of a full lantibiotic gene cluster) should be attempted.

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Appendices

Appendix 1 Preparation of selective agents

The selective agents used in this study were prepared as stock solutions in the appropriate solvent and sterilized by filtration with a 0.2 μm cellulose filter, when required. All the stock solutions prepared are summarized in Table S1.

Table S1: Summary of the stock solutions preparation for the selective agents used in this study. NR stands for non required. *Protect from light with foil paper.

Selective agent	Supplier	Stock solution	Final concentration	Solvent	Sterilization
Ampicillin	Sigma	100 mg/mL	100 μg/mL	Water	Filtration
Apramycin	AppliChem	50 mg/mL	50 μg/mL	Water	Filtration
Chloramphenicol*	BDH	25 mg/mL	12.5 μg/mL	Ethanol	NR
Kanamycin	Gibco	100 mg/mL	50 μg/mL	Water	Filtration
Tetracycline*	Sigma	20 mg/mL	10 μg mL	Ethanol	NR

Appendix 2 General Strains

The general bacterial strains used in this study are listed in Table S2.

Table S2: List of general strains used in this study with the reference to their genotype and supplier, when available. ATCC (American Type Culture Collection); DSMZ (German Collection of Microorganisms and Cell Cultures); FCUL (strains kindly provided by the Faculty Sciences of the University of Lisbon); INETI (Strain kindly provided by Dr. José C. Duarte; JIC (Jonh Innes Center); MUL (University of Lisbon Microorganims Collection); WWM (strains kindly supplied by Prof. Friedhelm Meinhardt from Westfälische Wilhelms-Universität Münster).

Strain	Source	Genotype/Characteristics
***E. coli* BL21 Gold**	Novagen	*E. coli* B F$^-$*ompThsdS*(rB$^-$ mB$^-$) *dcm*$^+$ Tet^r*galendA* Hte
***E. coli* BW25113**	JIC	*lacI*$^+$*rrnB*$_{T14}$Δ*lacZ* WJ16 *hsdR514*Δ*araBAD* AH33 Δ*rhaBAD* LD78
***E. coli* DH5α**	MUL	F$^-$*endA1glnV44* thi-1 *recA1relA1gyrA96deoRnupG*Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169, *hsdR17*(r_{K^-} m_{K^+}), λ$^-$
***E. coli* EPI300™ -T1^R**	Epicentre	F$^-$ mcrA Δ(*mrr-hsd*RMS-*mcr*BC) (Str^R) φ80d*lacZ*ΔM15 Δ*lacX74 recA1 endA1 araD139* Δ(*ara, leu*)*7697 galU galK* λ$^-$*rpsL nupG trfA tonA dhfr*
***B. licheniformis* I89**	INETI	Lichenicidin producer (Mendo *et al.*, 2004)
***B. licheniformis* ATCC 10716**	FCUL	Bacitracin producer
***B. licheniformis* MW3**	WWM	*B. licheniformis* DSM13 (*ΔhsdR1, ΔhsdR2*) (Waschkau *et al.*, 2008); Lichenicidin producer.
***B. halodurans* DSM 18197 (C-125)**	DSMZ	Haloduracin producer
***Micrococcus luteus* ATCC 9341**	MUL	Indicator strain

Appendix 3 General Vectors

The general vectors (plasmids and fosmids) also used in this study are listed in Table S3.

Table S3: List of general plasmids and fosmid used in this study, where MW refers to the molecular weigh of the vectors. Ampicillin (Amp); Apramycin (Apra); Chloramphenicol (Clo); Erythromycin (Erm); Kanamycin (Kan); Tetracycline (Tet). JIC (Jonh Innes Center); UC-USA (plasmids kindly provided by Prof. Daniel Portnoy from University of California, USA).

Vector	Source	MW	Selective marker	Observations
pLTV1	UC-USA	20.6 Kb	Amp^R, Clo^R, Erm^R, Tet^R	Amp^R, Clo^R, Erm^R associated with the Tn*917* transposon. ColE1 and oripE194^{TS}. oripE194 replicates at 32 ºC and segregates at 42 ºC (Camilli *et al.*, 1990).
pKSV7	UC-USA	6.9 Kb	Amp^R, Clo^R	*E. coli/Bacillus* shuttle vector. ColE1 and oripE194^{TS}. oripE194 replicates at 32ºC and segregates at 42 ºC (Smith and Youngman, 1992).
pET-15b	Novagen	5.7 Kb	Amp^R	Carries an N-terminal His•Tag® sequence followed by a thrombin site and three cloning sites.
pET-24a(+)	Novagen	5.3 Kb	Kan^R	Possess an N-terminal T7•Tag® sequence plus an optional C-terminal. His•Tag® sequence.
pCR®2.1	Invitrogen	3.9 Kb	Kan^R	Encodes the first 146 amino acids of β-galactosidase. Complementation in trans with the Ω fragment gives active β-galactosidase for blue-white screening.
pKD20	JIC	6.1 Kb	Amp^R	Low copy plasmid encoding the λ Red recombinase (γ, β, exo), which promote a greatly enhanced rate of recombination when using linear DNA. Possesses an optimized ribosome-binding site for efficient translation of γ and expresses γ, β, and exo from the arabinose-inducible P_{araB} promoter. It is also a temperature-sensitive replicon to allow for its easy elimination (Datsenko and Wanner, 2000).
pIJ733	JIC	4.3 Kb	$Apra^R$	The $Apra^R$ disruption cassette was cloned into the EcoRV site of pBluescript SK II (+). The cassette are flanked by FRT sites (FLP recognition targets) which allows FLP-mediated excision of the cassette (Gust *et al.*, 2003).
pCC2FOS™	Epicentre	8.2 Kb	Clo^R	*E. coli* F factor-based partitioning and single-copy origin of replication. *oriV* high-copy origin of replication. *cos* site for lambda packaging. *loxP* site for Cre-recombinase cleavage. Bacteriophage T7 RNA polymerase promoter flanking the cloning site.

Appendix 4 Preparation and transformation of chemically competent *E. coli* cells

A4.1 Preparation of competent cells by calcium-chloride method

Chemically competent cells were prepared using and adaptation of the procedure described by Sambrook and Russell (2001). The desired strain was inoculated in 10 mL of LB medium supplemented with the appropriated selective marker, overnight at 37 ºC, with aeration. The pre-culture (500 μL) was used to inoculate 50 mL of fresh LB medium supplemented with the appropriate antibiotic. The bacterial culture was grown at 37 ºC with aeration to an OD_{600nm} of approximately 0.3 and centrifuged at 4 ºC for 2 min at 6300 x g. The pellet was washed with 13 mL of ice-cold 0.1 M $MgCl_2$ and centrifuged as above. The cells were washed again with 25 mL of 0.1 M $CaCl_2$ solution, incubated on ice for 20 min and centrifuged as above. Finally the cells were resuspended in 1 mL of cryopreservation buffer, 50 μL were distributed in 1.5 mL microcentrifuge tubes and stored at -80 ºC until use. Before store cells should be immediately freeze on liquid nitrogen.

Solution:

Cryopreservation buffer: CaCl2 0.1 M, 15% (v/v) glycerol.

A4.2 Transformation

An aliquot of 50 μL of cells stored at -80 ºC were thawed on ice and the DNA was added (~5-100 ng of plasmid DNA or 5 μL of ligation reaction). The mixture was incubated in the 1.5 mL microcentrifuge tube on ice for 15 min and transferred to 42 ºC for 45 sec. The tube was immediately placed on ice for 2 min and 1 mL of LB medium was added. The cells were grown for 1 hour at 37 ºC with aeration. The cells were collected by centrifugation at 2300 x g for 1 min, resuspended in 100 μL of LB medium and finally were spread on LB agar plates containing the appropriate antibiotic. The plates were incubated at 37 ºC, overnight.

Appendix 5 Preparation and transformation of electrocompetent *E. coli* cells

A5.1 Preparation of electrocompetent cells

Electrocompetent cells were prepared by growing the desired strain in 10 mL of LB medium supplemented with the appropriated selective marker, overnight at 30 ºC, with aeration (160 rpm). This pre-culture (100 μL) was used to inoculate 10 mL of fresh LB medium containing 20 mM of $MgSO_4$ and the appropriate antibiotic. The culture was grown until an OD_{600nm} of ~0.4 at 30 ºC (due to the ori^{TS} of pKD20) with aeration and the cells were pelleted by centrifugation at 3300 x g for 5 min and 4 ºC. The medium was decanted and the cells resuspended in 10 mL of ice-cold 10% glycerol by gentle mixing. This wash step was performed twice, and finally the cells were resuspended in 100 μL of 10 % glycerol and kept at 4 ºC until use, since this procedure was always performed in the same day of transformation. The antibiotic used in the growth of strain *E. coli* BW25113/pKD20 was ampicillin and for *E. coli* BW25113/pKD20/pLIc5 were ampicillin (100 μg/mL) and chloramphenicol (12.5 μg/mL).

A5.2 Electroporation

The freshly prepared cells were transformed by electroporation using a Bio-Rad GenePulser II® equipment. For the procedure, 50 μL of cells were mixed with approximately 100-200 ng of DNA on ice. The mixture was transferred to a 0.2 cm ice-cold electroporation cuvette and the pulse parameters were set to: 200 Ω, 25 μF and 2,5 kV (the expected time constant was 4.5-4.9 ms). 1 mL of ice-cold LB medium was immediately added to the cells that were incubated at 30 ºC or 37 ºC (depending if replication or segregation of pKD20 was desired, respectively) for 1 hour, with aeration. The cells were centrifuged at 2300 x g for 1 min, resuspended in 100 μL of LB and spread in LB agar plates containing the appropriated selective markers. The plates were incubated overnight at 30 ºC or 37 ºC. The selection of *E. coli* BW25113/pKD20 strains transformed with the fosmid pLic5 was performed with ampicillin (100 μg/mL) and chloramphenicol (12.5 μg/mL) at 30 ºC. The selection of *E. coli* BW25113/pKD20/pLic5 strains possessing the desired gene interruption was performed with chloramphenicol (12.5 μg/mL) and apramycin (50 μg/mL) at 37 ºC.

Appendix 6 Extraction of plasmid DNA

A6.1 Mini-preparations

The routine extraction of plasmid DNA from *E. coli* was performed with the GeneJET™ Plasmid Miniprep Kit (Fermentas), according with manufacturer's instructions. Briefly, a bacterial culture was grown in LB medium with the appropriate selection agent, overnight at 37 °C with aeration (160 rpm). 5 mL of this culture was centrifuged at 6800 x g for 2 min and the cells were completely resuspended in 250 μL of Resuspension Solution. The Lysis Solution (250 μL) was added and the tube inverted until the solution became clear and viscous (no more than 5 min). Neutralization was performed by the addition of 350 μL of the Neutralizing Solution and by immediately mixing thoroughly the solution by inverting the tube. The lysate was centrifuged for 5 min at 12000 x g to pellet the cellular debris and chromosomal DNA. The supernatant was transferred to a GeneJET™ spin column, avoiding the transfer of the white precipitate and centrifuged for 1 min at 12000 x g. The flow-through was discarded and the column was placed back into the same collection tube. The DNA attached to the column was washed by the addition of 500 μL of Wash Solution followed by centrifugation for 1 min at 12000 x g. The flow-through was discarded and the column with the collection tube was centrifuged for an additional 1 min at the same speed to remove residual ethanol. Finally the column was transferred into a sterile 1.5 mL microcentrifuge tube. The plasmid DNA was eluted by the addition of 40 μL of sterile distilled water to the center of the column, incubation at room temperature for 2 min and centrifugation at 12000 x g for another 2 min.

A6.2 Maxi-preparations

If a higher concentration of plasmid DNA was required, the extraction was performed from an initial culture of 200 mL grown overnight at 37 °C with aeration in LB medium supplemented with the appropriate antibiotic. The procedure was adapted from the protocol described by Sambrook and Russell (2001).

The culture was centrifuged at 10000 x g for 6 min and the pelleted cells were resuspended in 6 mL of Solution I and incubated at room temperature for 5 min. To perform the cell lysis, 16 mL of freshly prepared Solution II containing 10 mg/mL of lysozyme (Roche) was added and the tube shacked vigorously in the horizontal position 5/6 times. The mixture was incubated on ice for 10 min and 12 mL of the alkaline Solution III were

subsequently added; the solution was gently mixed for 3 min. The mixture was incubated on ice for 10 min and the separation of plasmid DNA from the cell debris and chromosomal DNA was performed by centrifugation at top speed for 15 min at 4 °C. The supernatant containing the plasmid DNA was recovered avoiding the white precipitate of residual cell debris (using a Pasteur' pipette containing cotton at its bottom). The plasmid DNA was precipitated by addition of 0.6 volume of isopropanol to the filtered supernatant and incubated at room temperature for 15 min. The DNA was recovered by centrifugation at 8000 x g for 15 min and the pellet washed once with 5 mL of 70 % ethanol. The ethanol was removed by centrifugation in the same conditions as above and completely evaporated. The pellet was resuspended in 1 mL of TE containing 20 μg/mL of DNase-free RNase A (Roche), incubated at 37 °C for 1 hour and extracted three times with Phenol/CIA (Invitrogen). The DNA was precipitated by the addition of 1/10 volume of solution III and 0.6 volumes of isopropanol. The mixture was incubated at room temperature for 30 min and centrifuged at top speed for 15 min at 4 °C. The pellet was washed with 1 mL of 70 % of ethanol and a last centrifugation at top speed for 5 min was performed. The precipitated DNA was dried in the flow chamber for 15 min to evaporate the residual ethanol and resuspended in 100 μL of sterile distilled water.

Solutions:

Solution I: 50 mM Tris-HCl and 10 mM EDTA.
Solution II: 200 mM NaOH, 1% (w/v) SDS.
Solution III: 3 M potassium acetate, pH 5.5.
TE: 10 mM Tris-Cl, pH 8.0 and 1 mM EDTA. Final pH 8.0.

Appendix 7 Agarose gels handling

A7.1 Electrophoresis of DNA

Analysis of DNA was generally performed on agarose gel electrophoresis. The samples were mixed with 6X loading buffer in a proportion of 1:6 (v/v) and loaded in a 1 % agarose gel. The gel was prepared with 1X of TAE buffer (Bio-Rad) and EtBr (AppliChem) to a final concentration of 0.5 μg/mL that added before pouring the melted agarose in the running tray. In all the gels a DNA marker was included, either 0.5 μg of the DNA Ladder Mix (Fermentas) or 1 μg of the 2-Log DNA marker (New England Biolabs). Electrophoresis was generally performed at 5 V/cm for the desired time (normally between 1 – 1.5 hours, unless otherwise stated). The DNA was analized under UV light and the image acquired in the ATTO image acquisition system.

Solutions:

Loading Buffer 6X: 2.5 mg/mL of bromophenol blue, 2.5 mg/mL of xylene cyanol FF and 30% (v/v) glycerol. Stored at 4 ºC.

A7.2 Purification of DNA from agarose gels

The purification of DNA from agarose gels was performed using the QIAquick Gel Extraction Kit Protocol (Quiagen), according with the manufacturer's instructions. For that, the DNA fragment of interest was excised from the agarose gel with a clean scalpel and placed in a previously weighted 1.5 mL microcentrifuge tube. The gel slice was weighted and 3 volumes of Buffer Q1 were added to 1 volume of agarose (considering 100 mg as 100 mL). The tube was incubated at 50 ºC for 10 min (or at room temperature overnight) to completely dissolve the agarose slice. If the DNA fragment was smaller than 500 bp, 1 volume of isopropanol was added and well mixed. The sample was applied to a QIAquick spin column placed in a 1 mL collection tube and centrifuged at 12000 x g for 1 min. The flow-through was discarded and the column placed back to the collection tube. The DNA was washed with 750 μL of Buffer PE and the column centrifuged for 1 min at 12000 x g. The flow-through was discarded and the column centrifuged in the same conditions for 1 min to remove residual ethanol. The DNA-containing column was placed in a 1.5 mL clean microcentrifuge tube and the DNA eluted in distilled water. The elution volume (between 10 and 40 μL) was chosen according to the subsequent application. The eluted DNA was recovered after 2 min of incubation at room temperature by centrifugation for 2 min at 12000 x g. The sample was stored at -20 ºC until further use.

Appendix 8 Purification and concentration of PCR products and restrictions digestions

Purification and concentration of PCR products and DNA digestions were performed with the GeneJET™ PCR Purification Kit (Fermentas), according with manufacturer's instructions. Thus, 1 volume of Binding Buffer was added to 1 volume of the PCR or restriction digestion reactions. When the DNA fragment was smaller than 500 bp, this mixture was supplemented with 1:2 volumes of isopropanol. The solution was transfered to a GeneJET™ purification column, centrifuged for 1 min at 12000 x g and the flow-through was discarded. The DNA attached to the column was washed with 700 μL of Washing buffer and centrifuged 1 min at 12000 x g. The flow-through was discarded; the purification column placed back into the collection tube and centrifuged 1 min at 12000 x g to completely remove any residual wash buffer. Finally, the column was transferred to a clean 1.5 mL microcentrifuge tube and DNA eluted in distilled water (from 20 to 40 μL). After 1 min incubation at room temperature, the column/tube system was centrifuge for 2 min at 12000 x g. The collected DNA was stored at -20 ºC until use.

Appendix 9 Construction of the *B. licheniformis*I89 genomic library

In order to easily access to *B. licheniformis* I89 genome, a genomic library was constructed using the high-capacity vector pCC2FOS™ and accordingly with CopyControl™ Fosmid Library Production Kit (Epicentre) manufacturer instructions. The pCC2FOS™ fosmid is a single-copy vector, which increases the likelihood that all genome was represented. Some DNA sequences, if cloned into high-copy vectors like a cosmid, are unstable and, thus, could not be represented in the library.

A9.1 End-repair of HMW DNA

The end-repair of the high molecular weight (HMW) DNA was performed in a reaction containing 1X of end-repair buffer, 0.25 mM of dNTP mix, 1 mM of ATP, 40 μg of sheared HMW DNA and 8 μL of End-repair Enzyme Mix, in a final volume of 160 μL. The reaction was incubated for 45 min at room temperature. The inactivation of the enzyme was performed for 10 min at 70 ºC.

The reaction was loaded in a 1 % low-melt agarose gel, without the addition of EtBr. 100 ng of fosmid DNA control was loaded into the lanes adjacent to the HMW DNA. The gel was run at 3 5V for a period of 16 hours. After electrophoresis, the outer lanes containing the marker and a small portion of the lane containing the DNA were cut of and stained in an EtBr solution (0.5 μg/mL). Under the UV light, the position of the desired size of DNA was marked with a razor. The gel was reassembled and a slice 2 to 4 mm below the position of the fosmid control DNA was excised. The slice was weighted and stored in a 1.5 ml microcentrifuge tube at 4 ºC.

A9.2 Recovery of the HMW DNA

The blunt-end HMW DNA was extracted from the agarose using the GELase enzyme. Therefore, the agarose slice was melted at 70 ºC for 15 min, after what the tube was immediately transferred to 45 ºC. Add the appropriated volume of warmed GELase 50 X buffer (at 45 ºC) to a 1X final concentration. Add 1U of GELase enzyme preparation for each 100 μL of melted agarose, assuming that 1 mg of solidified agarose yields 1 μL of molten agarose. Incubate the reaction at 45 ºC for 1 hour. The enzyme was inactivated at 70 ºC for 10 min. 500 μL aliquots of the reaction were removed into sterile 1.5 mL microcentrifuge tubes and chilled on a ice bath for 5 min. The tubes were centrifuge at 10000 x g for 20 min and the supernatant was removed to a 1.5 mL tube, avoiding the

gelatinous pellet, if present. The DNA was precipitated adding 1/10 volume of 3M NaAc (pH 7.0) and 2.5 volumes of ethanol. After mixing by gentle inversion the tubes were incubated 10 min at RT. The precipitated DNA was centrifuged for 20 min at 10000 x g and the supernatant was aspirated. The pellet was rinsed twice with cold, 70 % ethanol and centrifuged for 20 min at 10000 x g. Finally, the pellet was dried 10 min in a flow-chamber, with the tube opened and resuspended in 10 μL of TE buffer. The DNA concentration was determined using Qubit® fluorometer (Invitrogen) accordingly with manufacturer's instructions.

A9.3 Ligation to pCC2FOS vector and packaging

Ligation of fractionated HMW DNA to pCC2FOS vector was performed in a 10 μL total volume reaction containing 0.5 μg of pCC2FOS DNA, 0.25 μg of HMW DNA, 1X Fast-Link ligation buffer, 1 mM ATP and 1 μL of Fast-Link DNA ligase. The reaction was incubated for 4 hours at room temperature and the enzyme inactivation was performed for 10 min at 70 °C. One tube of the MaxPlax lambda packaging extract was thawed on ice and 25 μL of this extract was transferred to a sterile 1.5 mL microcentrifuge tube and placed on ice. The remaining 25 μL of the extract was stored at -70 °C. 10 μL of the ligation reaction was added to the extract on ice and the solution was mixed by pipetting several times, avoiding however the introduction of air bubbles. The solution was incubated at 30 °C for 2 hours and the remaining 25 μL of MaxPlax lambda packaging extract was added. The reaction was incubated for another 2 hours at 30 °C. After the reaction was completed, the phage dilution buffer (PDB) was added to a final volume of 1 mL and mixed well and 25 μL of chloroform was added.

Solution:

PDB: 10 mM Tris-HCl [pH 8.3], 100 mM NaCl, 10 mM $MgCl_2$.

A9.4 Determination of the titer of the phage particles

Before plating the library, titer of the phage particles (packaged CopyControl Fosmid clones) was calculated in order to determine the number of plates and dilutions required to obtain the library needed. Therefore, serial dilutions of the 1 mL of packaged phage particles were performed in PDB. 10 μL of each dilution and 10 μL of the undiluted phage particles, were added, individually, to 100 μL of the prepared EPI300-T1R host cells and incubated 1 hour at 37 °C. The host cells were prepared as follows: on the day before use, 5 mL of LB broth supplemented with 10 mM $MgSO_4$ and 0.2 % maltose was inoculated with

EPI300-T1^R cells and grown at 37 °C. 500 μL of this culture was used to inoculate 50 mL of the same medium and the flask shaked at 37 °C until reached an OD_{600} of 0.9. The cells were stored at 4 °C until used.

The infected EPI300-T1R cells were spread on LB agar plates containing chloramphenicol (12.5 μg/mL) and incubated overnight at 37 °C. The colonies were counted and the titer of the packaged phage particles was calculated using the following formula:

$$\frac{(\#\ \text{ofcolonies})(\text{dilution factor})(1000\ \text{mL})}{\text{volume of phage plated (mL)}} = \text{titer in cfu/mL}$$

A9.5 Plating and selecting the I89 fosmid library

The determination of the number of fosmid clones required to reasonably ensure that any given DNA sequence of *B. licheniformis* I89 was represented in the library was calculated using the following formula:

$$N = \ln (1-P) / \ln (1-f)$$

Where *P* is the desired probability (expressed as a fraction); *f* is the proportion of the genome contained in a single clone; and *N* is the required number of fosmid clones. Therefore, the number of clones required to ensure a 99 % probability of a given DNA sequence of *B. licheniformis* I89(genome = 4.2 Mb) being contained within a fosmid library composed of 40-kb inserts was:

$N = \ln (1 - 0.99) / \ln (1 - [4 \times 10^4 \text{bases} / 4.2 \times 10^6 \text{bases}])$

$N = -4.61 / -9.55 \times 10^{-3}$

$N = 482$ clones

Based on the titer of the phage particles previously calculated and the number of clones necessary, the phage particles were diluted in PDB and mixed with the prepared EPI300-T1^{R}cells in a ratio of 100 μL of cells to 10 μL of diluted phage particles. The cells were incubated at 37 °C for 1 hour and spread on LB agar plates containing chloramphenicol (12.5 μg/ml). The plates were incubated at 37 °C overnight. The clones were picked and sequentially numbered to new LB agar plates containing the selective marker. All the clones were stored on 96-well plates at -80 °C.