Universidade de Aveiro
2012
Departamento de Química

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Lichenicidina: Regulação, Expressão e Bioengenharia em *E. coli*

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biotecnologia Molecular, realizada sob a orientação científica da Doutora Sónia Mendo, Professora auxiliar do Departamento de Biologia da Universidade de Aveiro e da Doutora Tânia Caetano, Investigadora em Pós-Doutoramento do Departamento de Biologia da Universidade de Aveiro

Dedicada aos meus queridos pais, Olinda e António,
e ao meu adorado irmão, João

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Agradecimentos

Foram muitas as pessoas que estiveram sempre presentes ao longo deste meu percurso académico, mas principalmente ao longo deste ano, que prometia ser difícil… e tal como prometeu, assim o cumpriu. No entanto, é quando alcançamos as metas difíceis que podemos sentir-nos orgulhosos do caminho que percorremos. E é também nesses momentos, que devemos agradecer a todos aqueles que tornaram o caminho um pouco mais fácil.

Assim, começo por agradecer às minhas orientadoras, Prof.ª Sónia Mendo e Dr.ª Tânia Caetano.

A si, Professora Sónia, agradeço por me ter recebido no seu laboratório (já há quase 2 anos e meio!); mais do que isso, no seu grupo, não só de trabalho mas também de almoço e de todas as outras pequenas coisas que fazem a diferença entre o local de trabalho e uma "família de trabalho". Um muito obrigada pelas dicas sempre úteis e pelo apoio sempre presente.

A ti, Tânia (e desculpa a falta da formalidade), por estares sempre lá, em corpo, espírito e ideias, para toda e qualquer dúvida, para puxares pelo meu barco quando eu achava que já não conseguia mais remar. E por acreditares sempre, sempre, sempre, que um dia eu iria conseguir, mesmo quando eu não acreditava que seria possível. Um muito obrigada por tudo. Mesmo.

Não posso, claro, deixar de agradecer a todas as meninas e meninos que ao longo deste ano estiveram comigo no nosso LBM, principalmente a Cátia, a Andreia e a Joana, pelos momentos de descompressão quando o dia corre menos bem e por aqueles pedacinhos da vossa experiência e das vossas ideias que sempre foram partilhando comigo para que pudesse ser melhor a cada nova apresentação, a cada nova etapa. Pelas críticas construtivas. Um muito obrigada por também fazerem parte deste trabalho.

Aos meus amigos e amigas do coração, por me arrastarem para tomar um café ou para dançar quando já deito fumo pelas orelhas; por aguentarem o meu mau humor quando estou com os nervos; por relerem o meu trabalho 500.000 vezes só para eu ficar descansada; por não desistirem mesmo quando eu o fiz. Um muito obrigada a todos e a todas por terem aparecido no meu caminho.

E por último, mas nem em último, aos meus queridos pais e irmão por serem sempre os primeiros a apoiarem-me em tudo, nas boas decisões e principalmente nas menos boas, pois é nestas ocasiões que mais preciso desse vosso apoio incondicional. Por me terem dado tudo o que podiam e por vezes o que não podiam para me verem crescer feliz em todas e cada etapa da minha vida. Por serem quem são, mesmo quando eu acho que não têm razão. Por me mostrarem que existem vários caminhos, mas me deixarem livre para escolher o que quero seguir. Por me levantarem sempre que tropecei em mais uma das muitas pedras do caminho. Por me acordarem para a vida e não deixarem que seja só trabalho. Um muito obrigada pelo dom da minha Vida. E em especial para o meu maninho, um muito obrigada por todas as parvoíces que sabias que me iam animar.

Obrigada a todos! ☺

Palavras-chave Lichenicidina, lantibioticos, expressão heteróloga, engenharia de péptidos, regulação da biossíntese, mutagénese aleatória

Resumo

A lichenicidina é um lantibiótico de classe II, naturalmente produzido por *B. licheniformis* I89. É constituída por dois péptidos denominados Bliα e Bliβ. Este lantibiótico foi o primeiro a ser expresso completamente *in vivo* num hospedeiro Gram negativo (*Escherichia coli*).

Neste trabalho, pretendeu-se avaliar o impacto da proteína LicR na biossíntese da lichenicidina usando um sistema de expressão heteróloga em *E. coli*. A estirpe de *E. coli* que não contem o gene *licR* parece apresentar uma maior produção de lichenicidin do que a estirpe que contem todo o conjunto de genes envolvidos na síntese da lichenicidin. Assim, LicR parece não apresentar qualquer função regulatória em *E. coli* ou esta não poderá ser descrita segundo os mecanismos habituais de regulação da produção de lantibióticos. Paralelamente um sistema de expressão foi construído para produzir cada um dos péptidos da lichenicidina separadamente, tendo sido comparados os níveis de produção de cada um dos péptidos. Este sistema foi usado com sucesso para produzir o péptido Bliβ mas não apresentando qualquer vantagem sobre os sistemas ao nível da produção. Finalmente, uma biblioteca de mutagénese do péptido Bliα foi construída em *E. coli* e os clones obtidos foram analisados; a maioria dos clones obtidos apresentou bioatividade reduzida ou nula contra *Micrococcus luteus*. Alguns destes clones foram sequenciados para determinar qual(ais) a(s) mutação(ões) presente(s) no gene *licA1*.

Keywords Lichenicidin, lantibiotics, heterologous expression, peptide engineering, biosynthesis regulation, random mutagenesis

Abstract

Lichenicidin is a class II lantibiotic, naturally produced by *Bacillus licheniformis* I89 strain. It is composed by two peptides: Bliα and Bliβ. This was the first lantibiotic to be fully produced *in vivo* using a Gram negative host (*Escherichia coli*).

Herein, the impact of LicR protein in lichenicidin biosynthesis was assessed, using an *E. coli* heterologous expression system. It was shown that the *E. coli* strain without the *licR* gene presented increased lichenicidin production, when compared with the strain containing the entire gene cluster. Thus, if LicR presents some regulatory function in *E. coli*, its role cannot be described according to the usually proposed regulation mechanisms involved in lantibiotic production. Also, an expression system was constructed to produce each lichenicidin peptide independently and this expression system was compared with other available systems in terms of production levels. The system was successfully used to obtain Bliβ peptide. However it did not show any advantage over the systems previously developed. Ultimately, a mutagenesis library of Bliα was constructed in *E. coli* and the clones were analyzed; the majority of the clones showed low or null bioactivity against *Micrococcus luteus*. Some of these clones were sequenced to determine which mutation(s) was present in the *licA1* gene.

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LIST OF ABBREVIATIONS

Amp	Ampicilin
Apra	Apramycin
cDNA	Complementary deoxyribonucleic acid
Clo	Chloramphenicol
dH_2O	Distilled water
dNTP's	Deoxynucleotide triphosphates
Dha	2,3-didehydroalanine
Dhb	2,3-didehydrobutyrine
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	1,4-Dithiothreitol
Kan	Kanamycin
Lan	Lanthionine
MeLan	Methyllanthionine
MRSA	Methicillin-resistant *Staphylococcus aureus*
MS	Mass Spectrometry
OD	Optical density
ORF	Open reading frame
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
Tc	Tetracyclin
UV	Ultra-violet
VRE	Vancomycin-resistant Enterococcus

CHAPTER I

GENERAL INTRODUCTION

Nowadays, search for novel compounds that can be useful in the treatment of bacterial infections is an important objective for the scientific community. The increasing capacity of bacteria to develop resistance leads to the inefficacy of the common antibiotics. Therefore, it is important to discover new compounds that are active against a large range of bacterial species (Donaghy, 2010, Gyssens, 2011).

In this context, a new type of antimicrobial peptides, the so-called lantibiotics, was discovered. These compounds are now under intense investigation in order to characterize and understand their biosynthesis and mode of action. They show activity against a large number of Gram positive bacteria, including the methicilin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci and oxacillin-resistant Gram positives (Bierbaum & Sahl, 2009, Field, *et al.*, 2010).

1.1 Lantibiotics

Lantibiotics are antimicrobial peptides, ribosomally synthesized by some Gram positive bacteria. They contain several unusual amino acids in their structure that result from enzyme mediated post-translational modifications (Figure 1). These peptides have particular interest because they can be much more potent against Gram-positive targets (including many antibiotic-resistant pathogens), than classical antibiotics, since they have the essential cell wall precursor lipid II as target. Another important feature is related with the fact that they are gene encoded (ribosomal synthesis), meaning that they can be more easily engineered to enhance their action (Field, *et al.*, 2010).

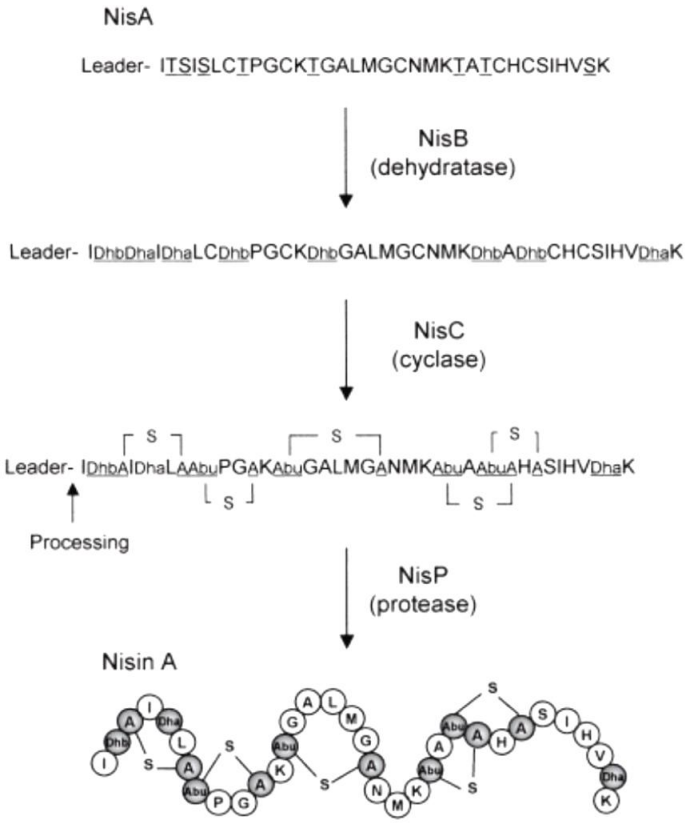

Figure 1 - Representation of the post-translational modifications involved in the biosynthesis of the lantibiotic nisin (Nagao, *et al.*, 2006).

Lantibiotics are characterized by the presence of post-translationally generated thioether linkages known as lanthionines (Lan) or β-methyllanthionines (MeLan), from where its denomination was originated (lanthionine-containing antibiotics) (Field, *et al.*, 2010). The active peptides and all the enzymes associated with their modification are gene-encoded. Their biosynthesis begins with the production of a prepropeptide. The prepropeptide (also known as prepeptide) is an inactive form of the lantibiotic, where none of the residues are modified (Figure 2). The prepropeptide can be divided in two regions: the N-terminal leader sequence and the C-terminal propeptide (Willey & Donk, 2007).

Figure 2 - Representation of the prepropeptide of the lantibiotic nisin (Willey & Donk, 2007). The leader sequence is represented in blue and the propeptide in red. Adapted from (Willey & Donk, 2007).

The leader sequence most probably promotes the transport of the peptide across the membrane by interacting with specific transporters. Moreover, it may be also important to keep the lantibiotic inactive until its secretion. Just immediately before or during the secretion process, the leader sequence is removed by a specific protease and the modified peptide becomes biologically active (Oppergard, *et al.*, 2007). Besides, the leader sequence seems to be necessary for the correct action of the modification enzymes. However, it is known that some peptide tags can be added to the leader sequence without affecting post-translational modifications (Nagao, *et al.*, 2006). The formation of the Lan and MeLan rings occurs exclusively in the propeptide region. The majority of the serines and threonines that are present in this area are enzymatically dehydrated to dehydroalanine (Dha) and dehydrobutyrine (Dhb), respectively (Figure 3). Sequentially, a cyclase catalyzes the regio- and stereoselective Michael additions of a cysteine onto Dha and Dhb amino acids, forming the Lan and MeLan thioether crosslinks correspondingly (Willey & Donk, 2007). The presence of this bridges convert the linear peptide into a polycyclic form, conferring not only structure and function to the peptide but also providing proteolytic resistance and increasing tolerance to oxidation (Field, *et al.*, 2010).

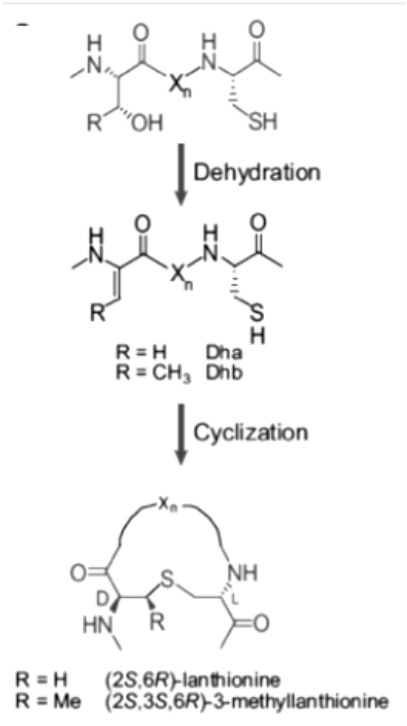

Figure 3 – Representation of the Lan and MeLan thioether ring formation in lantibiotics (Willey & Donk, 2007).

The general designation of the genes constituting the biosynthetic cluster is *lan* followed by a capital letter indicating the specificity of the gene. This general designation can be changed to a more specific nomenclature according to the lantibiotic that is being considered. For example, the genes involved in lacticin 3147 and nisin biosynthesis are designated as *ltn* and *nis*, respectively. The genes encoding all the enzymes involved in lantibiotic biosynthesis are usually found in clusters, which can be located in the chromosome (e.g. subtilin) or in mobile genetic elements such as transposons and/or plasmids (e.g. nukacin ISK-1) (Guder, *et al.*, 2000, Nagao, *et al.*, 2006, Willey & Donk, 2007). This localization seems to have no relation with the subtype grouping of lantibiotics (Nagao, *et al.*, 2006). All the gene clusters possess a *lanA* structural gene, which encodes the prepropeptide as well as other enzymes required for post-translational modification (*lanB*, *lanC*, *lanM*), leader peptide removal and peptide transport (*lanP*, *lanT*). Other genes involved in regulation (*lanR*, *lanK*) and/or immunity (*lanF*, *lanG*, *lanE*, *lanH*, *lanI*) may also be found within the biosynthetic cluster or in other closely related clusters (Guder, *et al.*, 2000, Willey & Donk, 2007).

1.1.1. Classification of lantibiotics

There are two main classification schemes used to group all the known lantibiotics: the Jung´s (Guder, *et al.*, 2000, Nagao, *et al.*, 2006) and the Pag & Sahl classifications (Pag & Sahl, 2002, Willey & Donk, 2007).

Pag & Sahl scheme will be adopted in the present work and is based on the pathway by which maturation of the peptide occurs as well as its biological activity (Pag & Sahl, 2002, Willey & Donk, 2007). According to this classification, the lantibiotics can be divided in three classes, which will be described in the following sections (Figure 4).

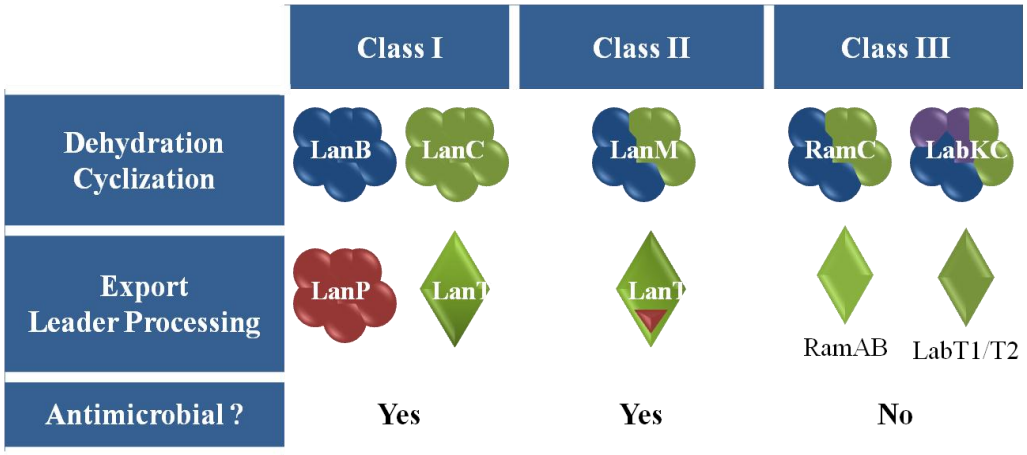

Figure 4 – Representation of the main differences between classes I, II and III lantibiotics, concerning the enzymes involved in modification, leader peptide processing and transport.

1.1.1.1 Class I

In class I lantibiotics, the prepropeptides are modified by two different enzymes: the LanB dehydratase and the LanC cyclase that mediates the thioether ring formation. The leader sequence removal and export of the peptide are performed also by two different enzymes: the subtilisin-like serine protease, LanP, and the ABC transporter, LanT. This class comprises the lantibiotics nisin and subtilin (Figure 5) and other related peptides.

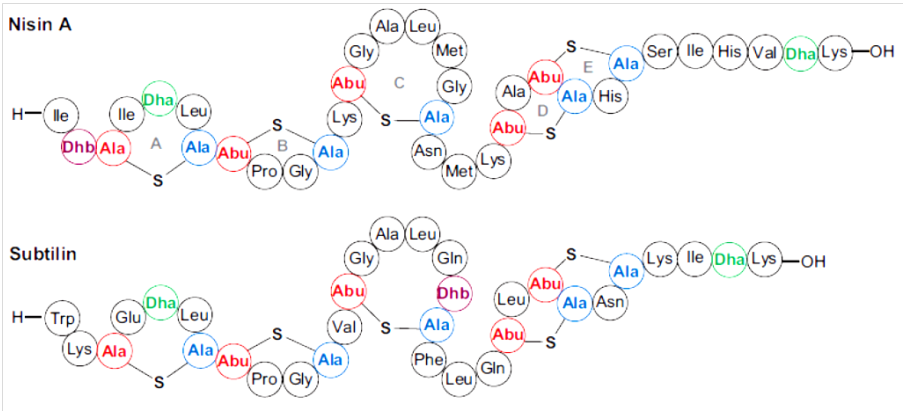

Figure 5 – Structures of representative examples of class I lantibiotics: nisin and subtilin (Willey & Donk, 2007).

1.1.1.2 Class II

In class II lantibiotics, the prepropeptides are modified by the LanM single enzyme, exhibiting both dehydratase and cyclase activities. LanM proteins do not show any homology to LanB proteins and have low sequence identity to LanC enzymes. Secretion and leader processing are performed by a single multifunctional protein that also shares the LanT designation. This class comprises the lantibiotics lacticin 481 and mersacidin (Figure 6), cinnamycin, duramycins and two-component lantibiotics (Willey & Donk, 2007, Field, *et al.*, 2010).

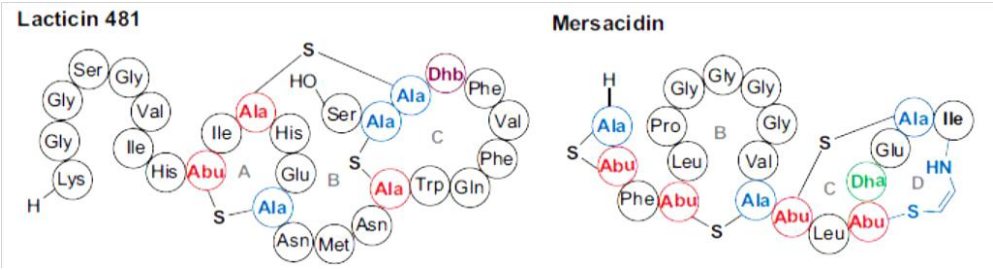

Figure 6 – Structures of representative examples of class II lantibiotics: lacticin 481 and mersacidin (Willey & Donk, 2007).

1.1.1.3 Two-component lantibiotics

The two-component lantibiotics are constituted by two peptides, which act synergistically to exhibit antimicrobial activity. Both peptides have a specific role in antimicrobial activity. Each of the peptides is encoded by its own structural gene and modified by separate LanM enzymes. However, a single LanT removes the leader sequence and secrete both peptides. In general terms, the two structural genes as well as

the two lanM genes are adjacent to each other in the same cluster, but in opposite directions (Figure 7) (Willey & Donk, 2007, Oman & van der Donk, 2009).

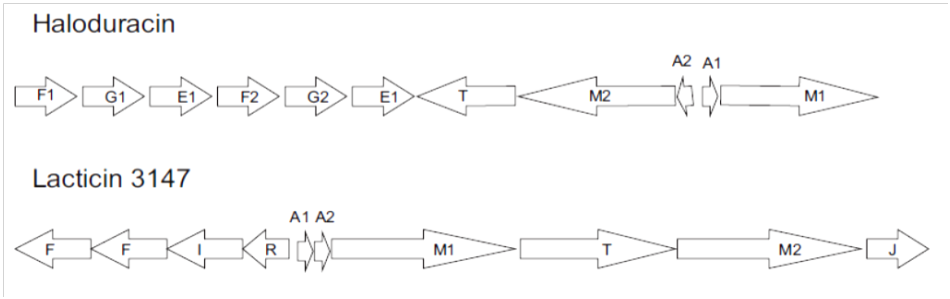

Figure 7 – Examples of the two-component lantibiotics haloduracin and lacticin 3147 gene clusters. Adapted from (Lawton, *et al.*, 2007) and (Willey & Donk, 2007), respectively. A1 and A2 represent the structural genes while M1 and M2 encode the respective modification enzymes; J encodes other enzyme which is necessary for the correct lacticin modification; R is a putative regulatory gene; T is the gene encoding the transporter protein; finally, F, G, E and I represent the immunity genes.

Historically, the unmodified peptides are designated LanA1 and LanA2, whereas the mature peptides are designated by the Greek symbols: Lanα and Lanβ. These peptides have diverse characteristics in common with one-peptide lantibiotics; they usually are cationic, containing hydrophobic and/or amphiphilic regions. Some examples of two-component lantibiotics include: plantaricin W, staphylococcin C55, cytolysin L, lacticin 3147 and haloduracin (Figure 8) (Willey & Donk, 2007, Field, *et al.*, 2010) and also the case of study, lichenicidin.

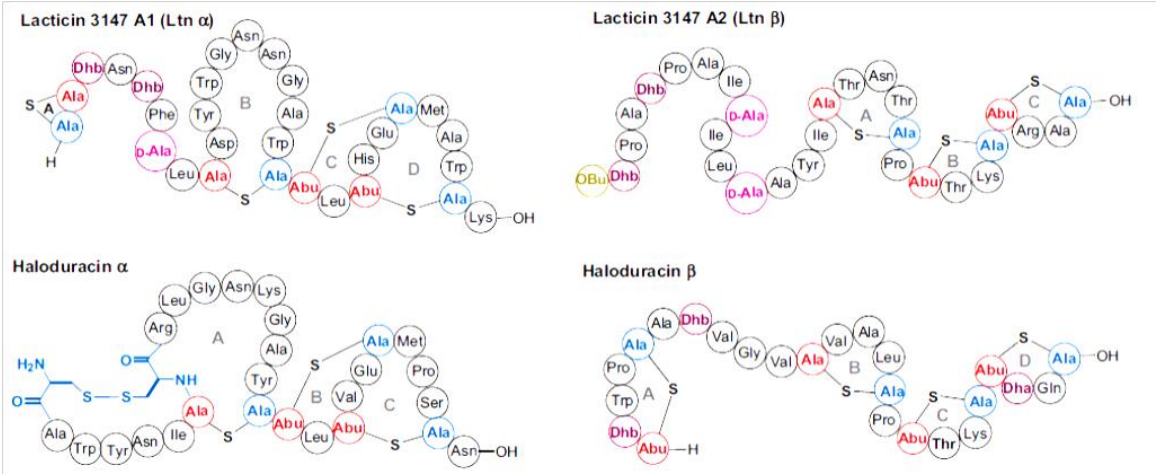

Figure 8 – Structures of representative examples of class II two-component lantibiotics: lacticin 3147 (A1 and A2) and haloduracin (α and β) (Willey & Donk, 2007).

The sequence homology between both peptides is low. In fact, the mature peptides of several two-component systems share structural and sequence homology with known

single-peptide lantibiotics but not with its own complementary one. Usually, mature α-peptides resemble the globular lantibiotic mersacidin with several fused thioether rings, while the mature β-peptides are typically elongated and more flexible (Oman & van der Donk, 2009).

1.1.1.4 Class III

This class comprises lanthionine-containing peptides that lack significant antibiotic activity; instead they perform another functions (e.g. as inhibition of phospholipase A2, biosurfactant activity, virulence factors) for the producing cell as is the case of AmfS produced by *Streptomyces griseus*, SapB (Figure 9) produced by *Streptomyces coelicolor* (Kodani, *et al.*, 2004) and SapT (Figure 9) produced by *Streptomyces tendae* (Kodani, *et al.*, 2005, Willey & Donk, 2007, Field, *et al.*, 2010). Labyrinthopeptins are also included in this class and can be distinguished by the presence of labionin, which is a carbacyclic, post-translationally modified amino acid derived from the activity of the enzyme LabKC on Ser-Xxx-Xxx-Ser-Xxx-Xxx-Xxx-Cys motifs in the corresponding propeptides (Field, *et al.*, 2010).

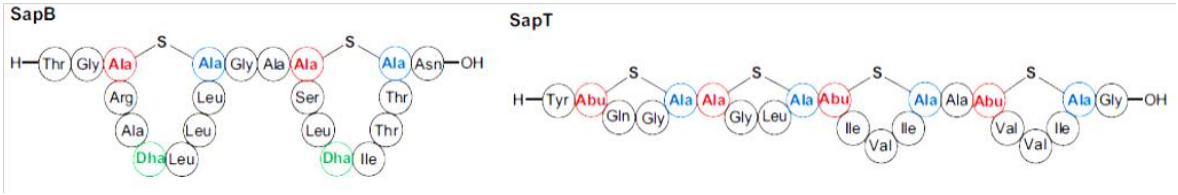

Figure 9 – Structures of representative examples of class III lantibiotics: SapB and SapT (Willey & Donk, 2007).

1.1.2 Biological activity of lantibiotics

As abovementioned, some lantibiotics are bactericidal at nanomolar concentrations against a variety of Gram positive bacteria, including the MRSA, (Willey & Donk, 2007, Bierbaum & Sahl, 2009). Lantibiotics have two main targets in the bacterial cell: the cell-wall intermediate lipid II and the cytoplasmic membrane. Nisin, a class I lantibiotic, exerts its activity on both of these components: its two N-terminal thioether rings form a binding pocket also called the pyrophosphate cage, which is stabilized by hydrogen bonds. This cage envelops the undecaprenyl pyrophosphate moieties of the lipid II molecule. After binding to lipid II, the positively charged C-terminus is able to insert into the membrane, oligomerize and form a pore that contains eight nisin molecules and four lipid II molecules (Figure 10) (Bierbaum & Sahl, 2009).

Considering the class II two-component lantibiotics, each of the peptides individually can have some antimicrobial activity, but at low levels. However, high activity (from pico to nanomolar concentrations) is only reached if the two-peptides are combined, since they act synergistically to inhibit the growth of other Gram positive bacteria. Their general mode of action is illustrated by the lacticin 3147 lantibiotic (Ltnα and Ltnβ): it has been proposed that the α-peptide binds to lipid II thereafter, the β-peptide is able to recognize this complex and bind it, subsequently inserting into the cytoplasmic membrane and forming a pore (Figure 10) (Bierbaum & Sahl, 2009)

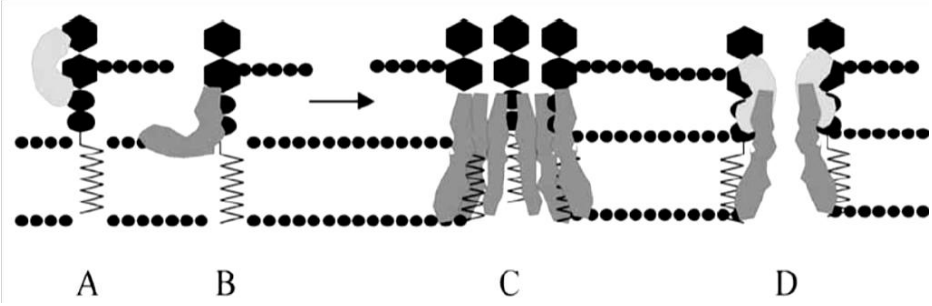

Figure 10 – General description of the mode of action of lantibiotics. A cytoplasmic membrane (black circles) with the lipid II attached is represented. In (A), the lantibiotic molecule binds to the head group of lipid II; in (B) nisin attaches to lipid II with its N-terminus and subsequently inserts into the membrane and forms a pore consisting of 4 lipid II and 8 nisin molecules (C); in (D) pore formation by a two-peptide system is shown: the α-peptide binds to lipid II and the β-peptide forms the pore (Bierbaum & Sahl, 2009).

However, as referred, not all the lantibiotics are antimicrobials. For instance, the two-component lantibiotic cytolysin, not only targets other Gram positives, but also functions as a virulence factor, lysing erythrocytes and polymorphonuclear leukocytes (Cox, *et al.*, 2005, Willey & Donk, 2007). In class III lantibiotics, potent inhibitors of phospholipase A2 (cinnamycin) can be found, but also peptides that can increase the chloride secretion in lung epithelium (duramycin) (Marki, *et al.*, 1991, Willey & Donk, 2007). Moreover, SapB and SapT are both hydrophobic and surface active peptides, function as biosurfactants, that release the surface tension at the colony-air interface (Kodani, *et al.*, 2004, Kodani, *et al.*, 2005, Willey & Donk, 2007).

1.1.3 Regulation of lantibiotic biosynthesis

In most cases, lantibiotic production is an adaptive advantage, and so, it is regulated by the presence of other microorganisms or other adverse environmental conditions. It could also be useful for the uptake of homologous DNA when associated with competence development, by selectively targeting non-competent cells of the same strain (Willey & Donk, 2007). Lantibiotic production is often regulated with other cellular events and takes place usually in the late exponential growth phase (Chatterjee,

et al., 2005, Willey & Donk, 2007). Biosynthesis of several lantibiotic and nonlantibiotic peptides seems to be regulated by typical bacterial two-component regulatory systems using the molecule itself as trigger, functioning as quorum sensing molecules (Guder, *et al.*, 2000).

The regulation of several lantibiotics biosynthesis has been studied. For instance, autoregulation of nisin and subtilin is performed by sub inhibitory concentrations of these class I lantibiotics in the extracellular environment. This was found to initiate a kinase/response regulatory signal transduction system that increments the transcription of biosynthetic and immunity genes. Usually these mechanisms are active during mid-exponential growth of the cell and they reach a peak of production at the log- to stationary-phase transition (Willey & Donk, 2007). In the case of subtilin, regulation depends on the transcription of the *spaRK* operon, which encodes the response regulator (*spaR*) and the signal kinase (*spaK*). The transcription of this operon is also regulated and dependent on the alternative sigma factor, σ^H, which is regulated at transcriptional and translational levels (Stein, *et al.*, 2002, Willey & Donk, 2007).

Concerning *Bacillus* sp. HILY-85 strain, it seems to coordinately regulate mersacidin (class II lantibiotic) biosynthesis with other stationary-phase events and in fact, the peptide is not produced until the beginning of the stationary phase. Contrarily to subtilin, this process is σ^H-independent. It was also observed that mersacidin gene cluster encodes two different response regulators MrsR1 and MrsR2/MrsK2. MrsR2/MrsK2 complex regulates the transcription of the self-immunity genes, whereas MrsR1 is exclusively involved in the production of the peptide itself. Synthesis of mersacidin seems not to be autoregulatory but controlled by a so-called orphan response regulator without a dedicated kinase (Schmitz, *et al.*, 2006, Sass, *et al.*, 2008). Other examples of this system include the lantibiotics lacticin 3147, mutacin II, epidermin and SapB (Willey & Donk, 2007).

The regulation of epidermin, a lantibiotic produced by *Staphylococcus epidermidis*, is in part controlled by global cellular stress response regulators and biofilm formation. The EpiQ protein, encoded in the epidermin biosynthetic cluster, regulates the transcription of the *epiA* structural gene. However, EpiP production, necessary for the removal of epidermin leader sequence, is under the control of the global regulatory system *agr* (Willey & Donk, 2007).

Another example of regulation mechanisms can be found in the production of lacticin 481 by *Lactococcus lactis* strains, which is regulated by two promoters, P1 and

P3; the expression of the genes under the control of these promoters is stimulated by acidification of the medium due to the presence of lactic acid. The co-transcription with a universal stress-like protein and a multidrug transporter leads to an increase of acid tolerance (Willey & Donk, 2007).

Focusing on the mechanism that regulates the production of the two-component lantibiotics, the best well-characterized system is that of cytolysin ($CylL_S$ and $CylL_L$). Cytolysin works as an *Enterococcus faecalis* virulence factor and is regulated by a quorum sensing mechanism that is dependent on the density of eukaryote cells. In the absence of target cells, cytolysin production is repressed by CylR1 that dimerizes and binds specifically to an inverted repeat that overlaps the -35 region of the cytolysin operon promoter (Figure 11a). However, a low-level of cytolysin peptides is ensured by basal transcription of the biosynthetic cluster. The two peptides form an insoluble complex that has neither regulatory nor cytolytic activity. In the presence of the target cells, $CylL_L$ will bind preferentially to phosphatidylcholine: cholesterol lipid bilayers and will no longer bind with $CylL_S$. Thus, this peptide will accumulate in the extracellular environment and will lead to an increase in cytolysin expression level (Figure 11b). The mechanism of derepression is still not completely understood, but it is known that a second membrane binding protein, called CylR2, is also involved but with unknown function. Overall, it is clear that this mechanism allows *E. faecalis* to use a single peptide to probe the environment for cytolysin targets and induce its production only when it is needed, leading to an economy of regulation (Coburn, *et al.*, 2004, Willey & Donk, 2007).

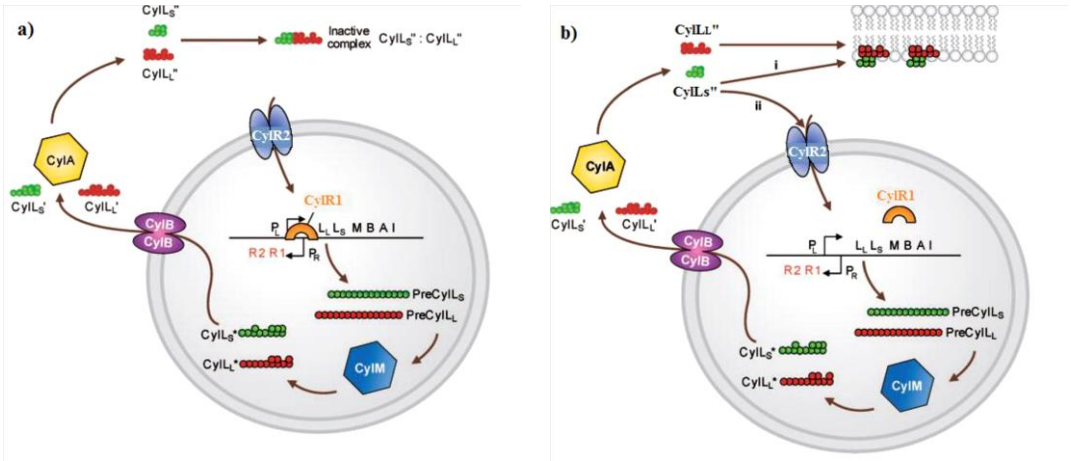

Figure 11 – Regulation mechanism of cytolysin biosynthesis in the absence (a) and presence (b) of eukaryotic target cells (Willey & Donk, 2007).

1.1.4 Characterization of the lantibiotic lichenicidin

Bacillus licheniformis I89 is a Gram positive endospore-forming bacterium found in the soil that produces a peptide with activity against Gram positive bacteria (Mendo, *et al.*, 2004). Other microorganisms that also belong to this *Bacillus* group have been described as producers of proteases, amylases, antibiotics and surfactants, which are considered biotechnologically important compounds. Among these compounds produced there are antimicrobial peptides that can be nonribosomally or ribosomally synthesized (Caetano, *et al.*, 2011).

Considering the ribosomally synthesized peptides possessing antibacterial activity, it was found that *B. licheniformis* I89 naturally produces a two-component lantibiotic (class II): lichenicidin (Figure 12).

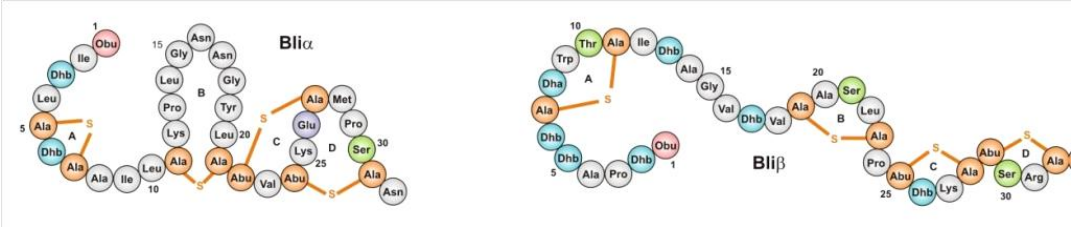

Figure 12 – Representation of Bliα and Bliβ structures (Caetano, *et al.*, 2011)

Lichenicidin is active against MRSA and *Listeria monocytogenes*. Apparently, its mechanism of action involves the interaction of both peptides with the membrane molecule lipid II, leading to the formation of pores in the bacterial membrane in such a way that the targeted microorganism loses its viability (Shenkarev, *et al.*, 2010). According to the definition of two-component lantibiotics, if only one of the peptides is produced, there will be no antimicrobial activity, but the activity can be restored if the complementary peptide is supplied by cross feeding (Caetano, *et al.*, 2011).

All the genes necessary for the lichenicidin synthesis, regulation and immunity are encoded in the *lic* gene cluster (Figure 13) (Rey, *et al.*, 2004, Dischinger, *et al.*, 2009, Caetano, *et al.*, 2011).

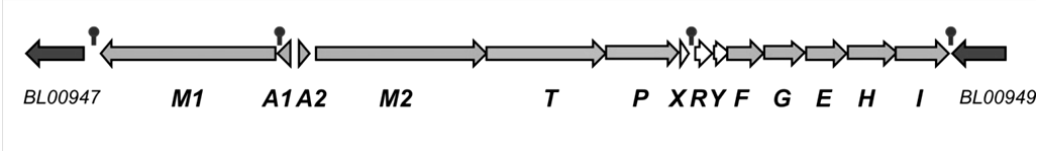

Figure 13 – Representation of the *lic* gene cluster organization, according to the genome annotation for *Bacillus licheniformis* ATCC 14760 (Caetano, *et al.*, 2011).

Since the original producer *B. licheniformis* I89 has low transformation efficiency, it was difficult to study the function of the genes present in the *lic* cluster. Thus, the

complete gene cluster for lichenicidin production was introduced in the Gram negative host *Escherichia coli*. In this heterologous system, lichenicidin production was achieved (Caetano, *et al.*, 2011). Usually, *E. coli* is the host microorganism of choice to be used for heterologous expression due to its characteristics for genetic manipulations, handling, costs and generation time. The two lichenicidin peptides are encoded by two different structural genes (*licA1* and *licA2*) that after their expression are modified by two different proteins LicM1 and LicM2, respectively. The peptides become biologically activate after the removal of the leader sequence and are transported to the extracellular environment by a single multifunctional protein called LicT that contains an ABC transporter and a protease domain (Caetano, *et al.*, 2011). After all the post-translational modifications, LicA1 and LicA2 became mature lantibiotics and are designated as Bliα and Bliβ (Figure 12), respectively. The *lic* biosynthetic cluster also includes other genes, for instance *licP*, which encodes a serine protease acting exclusively in the activation of Bliβ peptide. *licR* encodes a putative regulatory protein and *licY* encodes a protein with unknown function. In *E. coli*, LicR and LicY seem to be involved exclusively in the production of Bliα or Bliβ, respectively. *licX* encodes a small uncharacterized protein with unknown function that does not affect lichenicidin production in the heterologous expression host. *licFGEHI* are the so-called immunity genes, where *licFGE* encode an ABC transporter, *licI* encodes an individual immunity protein and *licH* encodes an auxiliary protein essential for the correct assembly of the functional ABC transporter. The presence of these genes is not essential for the lichenicidin production in *E. coli* (Caetano, *et al.*, 2011, Caetano, *et al.*, 2011).

1.1.5 Bioengineering of lantibiotics

The gene encoded nature of lantibiotics allowed the development of mutagenesis systems to produce novel structural variants. These systems can be used not only to reveal information about structure-function relationships but also to enhance chemical and antimicrobial properties of lantibiotics and even their rational design. Usually *in vivo* bioengineering of the structural peptide(s) is performed in the original producer or closely relatives once there are multiple genes required for lantibiotic synthesis and immunity (Kuipers, *et al.*, 1996, Field, *et al.*, 2007, Nagao, *et al.*, 2007).

Different techniques can be used to perform such modifications, namely site-directed mutagenesis and random mutagenesis. Site-directed mutagenesis ensures the replacement of a single specific amino acid but it is time consuming and unsuitable for

random mutagenesis approaches. Those strategies established/confirmed the importance of specific residues both in the structural peptides and respective leader sequences (Field, *et al.*, 2007). Several lantibiotics have already been mutated by site-directed mutagenesis approaches, for instance nisin A, nisin Z, gallidermin, epidermin and Pep5 (Kuipers, *et al.*, 1996). Nukacin ISK-1 was also object of bioengineering studies but using other methodologies for the insertion of mutations (Nagao, *et al.*, 2007).

Random mutagenesis is a useful tool to generate optimized, non active or altered proteins due to the insertion of random alterations in the DNA that encodes the protein. It can generate a large number of variants, some of which will produce a desired effect in the protein (Nicholl, 2008, Minamoto, *et al.*, 2012). This approach is advantageous when comparing to the alternative site-directed mutagenesis as prior knowledge of the functional importance of each residue is not necessary; in fact, this technique requires efficient screening methods than previous sequence information. For the same reason, it could be very difficult to associate the improved phenotype with the underlying genotype (Nicholl, 2008, Minamoto, *et al.*, 2012, Zhang, *et al.*, 2012).

Several methods to perform random mutagenesis are known such as error-prone PCR (epPCR), UV irradiation or chemical mutagenesis and saturation mutagenesis. epPCR is the most widely used for *in vitro* mutagenesis and will be used in the present work. It is usually performed using DNA polymerases without proof-reading activity (Minamoto, *et al.*, 2012).

The mutation frequency is controlled by adjusting the initial amount of target DNA and/or the number of thermal cycles and can be determined for an amplification reaction considering the error rate of the DNA polymerase and the number of duplications during PCR (Emond, *et al.*, 2008). The mutation frequency must be adapted to a particular application. For instance, to analyze protein structure-function relationships, the desired mutation frequency is one amino acid change (1–2 nucleotide changes) per gene (Vartanian, *et al.*, 1996), whereas to obtain proteins with improved activities it is necessary to isolate them from highly mutagenized libraries, exhibiting 20 mutations per gene (Daugherty, *et al.*, 2000). Mutant libraries can be constructed at various mutagenesis frequencies: low mutagenesis frequency offer a high probability of functional sequences and a low probability of beneficial mutations (increased activity) while high mutagenesis frequency leads to a high probability of lethal mutations with a high probability of unique sequences, that are more difficult to identify. Usually several

libraries are performed combining different mutation frequencies according to the intended results (Ye, *et al.*, 2012).

1.2 Objectives of this thesis

The work developed in the present thesis has its main focus in the characterization of the regulation mechanism of lichenicidin biosynthesis and its heterologous expression under the control of *E. coli* determinants. Additionally a system of peptide bioengineering to produce mutants with significant altered bioactivity was attempted. To achieve these goals, several studies were conducted and constituted the following tasks:

- Determine the role of LicR protein in lichenicidin biosynthesis regulation, using either the heterologous expression system in an *E. coli* host and the original producer, *B. licheniformis* I89.
- Understand if the production of each lichenicidin peptides can be achieved independently, using only their own essential genes and under the control of an *E. coli* promoter.
- Compare the yield of production and bioactivity of the different biosystems available for the production of lichenicidin in order to understand which of them is the most efficient.
- Produce *E. coli* mutants with increased and decreased or no activity using a peptide bioengineering approach: random mutagenesis.

CHAPTER II

INVOLVEMENT OF LICR IN THE BIOSYNTHESIS OF BLIα PEPTIDE

2.1 Background

Biosynthesis of lantibiotics is a process that requires a significant amount of energy and consequently it must be strictly controlled (Bierbaum & Sahl, 2009). The system generally involved in lantibiotic regulation is composed by two proteins: the receptor-histidine kinase LanK and the transcriptional response regulator LanR. The first one is the responsible for monitoring external environmental signals, inducing a response cascade involving the phosphorylation of LanR that is intracellularly located. LanR will then mediate the final response, usually by changing gene expression (Dale & Park, 2004). Regarding two-component lantibiotics biosynthesis regulation, the most studied case is cytolysin, as mentioned in the previous chapter (see section 1.1.3).

The analysis of LicR sequence showed higher sequence homology with helix-turn-helix (HTH) XRE family-like proteins (Figure 14), including the HalR protein (encoded in the two peptide lantibiotic haloduracin gene cluster) and also with other regulator proteins from strains belonging to the *Bacillus* genus. The HTH_XRE proteins are a family of DNA binding proteins, normally associated with the regulation of gene transcription (Wintjens & Rooman, 1996).

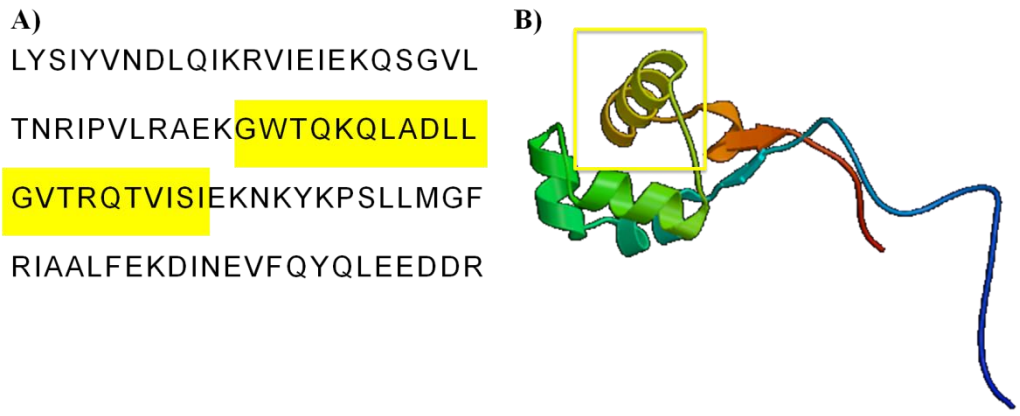

Figure 14 - (A) Sequence of LicR protein with the predicted HTH motif highlighted (yellow). **(B)** LicR secondary structure according to the prediction of the $(PS)^2$ Protein Structure Prediction Server (Chen, *et al.*, 2006)

In a previous study, using the heterologous host *E. coli*, it was observed that the deletion of *licR* gene from the lichenicidin gene cluster resulted in the absence of Bliα peptide (Caetano, *et al.*, 2011). Thus, based on LicR sequence homology, it was hypothesized that LicR could be involved in the regulation of *licA1* and/or *licM1* transcription, once these genes are directly implicated in the production of Bliα peptide but not in Bliβ's.

To confirm this hypothesis, the objective of this chapter was to compare the *licA1* and *licM1*expression levels of the *licR* knockout mutant (*E. coli* BLic5ΔR) with those of the control strain (*E. coli* BLic5 containing *licR* gene). As it will be explained, deletion of *licR* in the lichenicidin original producer *B. licheniformis* I89 was also attempted.

2.2 Results and Discussion

2.2.1 Analysis of the *licA1M1* promoter region

Taking in account that LicR as a putative regulatory protein and considering the fact that the deletion of *licR* in *E. coli* lead to the absence of Bliα peptide without affecting Bliβ, it seemed reasonable to assume that LicR should be involved in the regulation of *licA1* and/or *licM1* expression.

The *licA1M1* nucleotide region was characterized regarding the presence of putative promoters, ribosome-binding sites (RBS) and terminators (Figure 15). As shown in the figure, it was possible to identify a promoter upstream to the *licA1* gene, containing both -35 and -10 boxes (P_{licA1} promoter). However, such a genetic structure could not be identified into the intergenic region between *licA1* and *licM1*. Also two putative RBS were identified upstream of these two genes. Moreover, the search for terminators within the sequence was performed using the web server Transcriptional Terminators Prediction. Only results presenting the same orientation of both genes were considered and only the first one after the stop codon of the coding sequence. Considering all these restrictions, two terminators within the sequence were found: one after *licA1* coding sequence and another after *licM1*. Both seem to be Rho-independent termination signals, since they present an inverted repeat sequence GC-rich followed by four or more adenines. During transcription, the inverted repeat sequence allows RNA to form a stem-loop structure that causes the release of the RNA from DNA polymerase, which stops the transcription.

In conclusion, this analysis suggests that *licA1* and *licM1* expression should be under the control of the P_{licA1} promoter. Thus, *licA1* and *licM1* are transcribed together. Nevertheless one putative terminator was identified after each of these genes, thus emphasizing the importance of studying the expression levels of *licA1* and *licM1* independently.

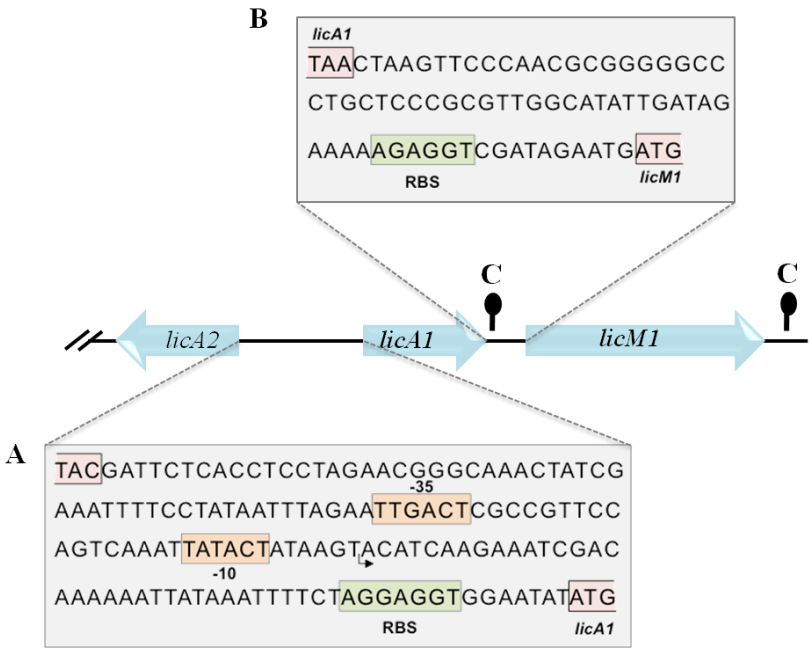

Figure 15 – Representation of lichenicidin gene cluster region containing *licA2*, *licA1* and *licM1* genes. (A) Region prior to *licA1*; (B) Intergenic region between *licA1* and *licM1*; (C) terminators according to Transcription Terminator Prediction web server; -35 and -10 (Pribnow box) – transcription regulatory regions; the arrow marks the trascription initiation site (according to BPROM software); RBS – ribosome binding site.

2.2.2 Analysis of total RNA extracted from BLic5 and BLic5ΔR strains

The expression levels of *licA1* and *licM1* genes in the presence and absence of the putative transcriptional regulator *licR* was predicted to be performed using RT-qPCR.

After extraction of total RNA from BLic5 and BLic5ΔR strains, the possible contamination with DNA was evaluated by PCR. In the reactions, primers targeting the *licA1* and *licM1* complete genes were used. Also, two positive controls, consisting of colonies of BLic5 and BLic5ΔR strains, were always included. It was observed that the extraction procedure was efficient regarding the absence of total DNA, since none of the two genes were amplified when total RNA was used as template. As expected, amplification was always observed for *licA1* and *licM1* genes for the positive controls. However, the analysis of the agarose gel revealed a difference in the *licM1* amplification: the amplicon of BLic5ΔR presented higher molecular weight than that of BLic5 (Figure 16).

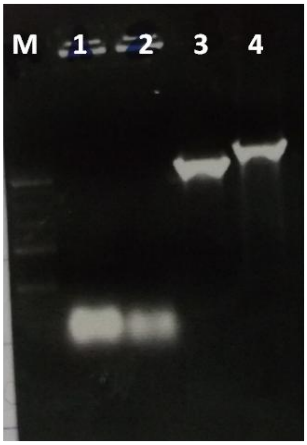

Figure 16 – Electrophoresis gel representing the licM1 amplification of total RNA (lines 1 and 2) and the positive controls (lines 3 and 4); M – LadderMix GeneRuler; 1 – BLic5 total RNA; 2 – BLic5ΔR total RNA; 3 – BLic5 colony; 4 – BLic5ΔR colony

Subsequently, the same reaction was performed including also a colony of the original lichenicidin producer *B. licheniformis* I89 strain, which allowed concluding that *licM1* amplification for BLic5 and I89 strain presented the same molecular weight (data not shown). So, the size of fragment obtained for BLic5ΔR was bigger that the expected. This result suggested that *licM1* gene should possess an insertion in the *licR* knockout strain. Therefore, the RT-qPCR analysis was not performed and a new BLic5ΔR knockout strain was constructed.

2.2.3 Comparison of lichenicidin production between BLic5 and BLic5ΔR strains

To obtain a new BLic5ΔR knockout strain, the *licR* gene was deleted from the pLic5 fosmid according with the procedure described in section 2.4.3. The obtained fosmid (pLic5ΔR) was investigated for the correct *licM1* amplification. Since an amplicon of the same size as *licM1* was obtained with I89 strain total DNA and with pLic5ΔR DNA, the fosmid was transformed in *E. coli* BL21-Gold(DE3) resulting in the correct BLic5ΔR strain.

The production of lichenicidin peptides by BLic5ΔR was first evaluated by colony bioassay. The plates showed that BLic5ΔR strain was able to produce both lichenicidin peptides, since an inhibition area against *M. luteus* was observed (Figure 17). This result demonstrated that in the heterologous expression system previously described by Caetano *et al*. (2011) *licR* is not essential for Bliα production. Thus, the results previously obtained were due to LicM1 inactivity, instead of the *licR* absence.

Figure 17 – Antibacterial activity exhibited by BLic5 and the new BLic5ΔR strains.

The colony-bioassay indicated that both Bliα and Bliβ were produced by BLic5ΔR strain. However, using this technique, a comparison of the production levels with that of the control strain (BLic5) is not possible. Thus, to investigate the impact of *licR* absence on lichenicidin production levels, liquid cultures of both strains were performed in triplicate and the lantibiotic was extracted with 1-butanol. After evaporation, the bioactivity of the samples was investigated and quantified using arbitrary units (section 3.5.3). The same extracts were analyzed by HPLC-ESI-MS to detect and measure more accurately the amounts of Bliα and Bliβ present.

The bioactivity results showed that there were no significant differences between both strains (Figure 18) since the absence of activity was observed approximately at the same dilution for BLic5 and BLic5ΔR. This could indicate that *licR* has no influence in the lichenicidin biosynthesis process, when the *lic* gene cluster is expressed in *E. coli*.

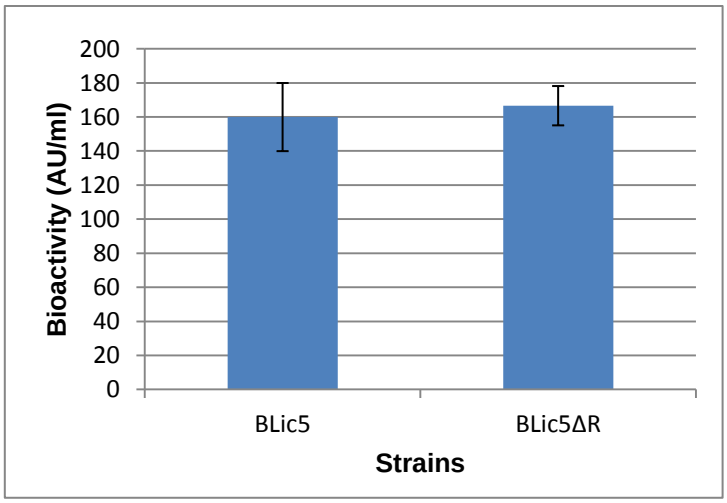

Figure 18 - Quantification of BLic5 and BLic5ΔR bioactivity against *M. luteus*. The AU/mL was calculated using a series of dilutions and considering the last well that showed inhibition.

However, these results were compared and confirmed by the HPLC-ESI-MS analysis. Using this technique, the concentration of both lichenicidin peptides was determined (Figure 19).

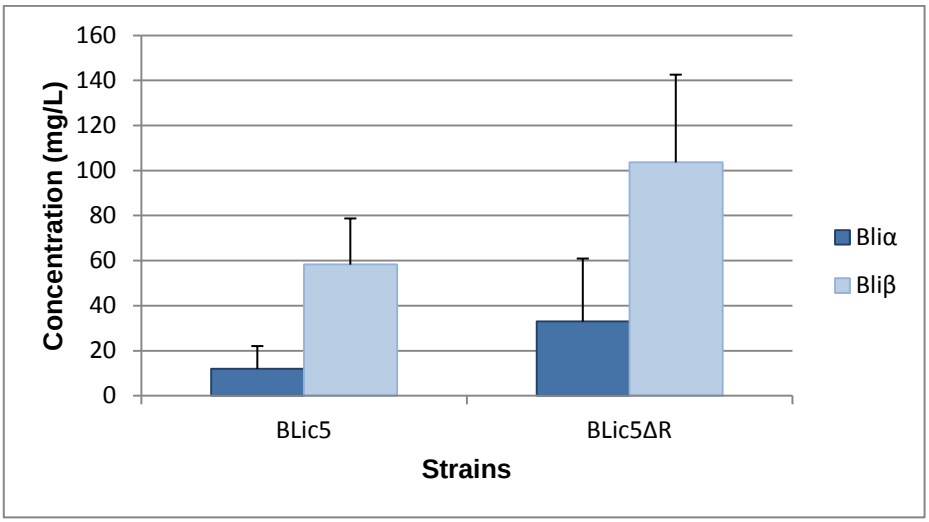

Figure 19 – Quantification of Bliα and Bliβ in production both BLic5 and BLic5ΔR by HPLC-ESI-MS.

Contrarily to what was observed in the bioassay analysis, the mass spectrometry results seem to indicate that BLic5ΔR produces more lichenicidin than BLic5. This indicates that the absence of LicR can be somehow advantageous for lichenicidin production in *E. coli*. It is important to notice that in the bioassay the synergistic effect of both peptides is analyzed, while in MS analysis each peptide is analyzed independently making this method more suitable and the results more accurate. It is known that regulation mechanisms are different in Gram negative and Gram positive, especially because the trigger molecules of each system. In fact, once in Gram positive bacteria, the lantibiotic is the trigger molecule itself, in Gram negative bacteria there are other factors that mediate the regulatory mechanism. For example, a study using colicin E1 (antimicrobial peptide naturally produced by some *E. coli* strains) suggests that under anaerobic control the transcriptional expression level of this peptide was increased (Eraso & Weinstock, 1992). Also other factors can regulate gene expression, such as nutrient depletion, pH changes or production of metabolites/inducers (Kuhar & Zgur-Bertok, 1999). Indeed, a common regulation mechanism of diverse cellular processes of Gram negative bacteria is mediated by N-acyl-homoserine lactone molecules through a quorum-sensing mechanism. Those lactones can diffuse across the cell membrane and enter the other cells where they interact with the regulatory protein

directly; if the lactone concentration is sufficient, the activated regulatory protein will switch on the target genes (Dale & Park, 2004) .

Thus, the results obtained for *licR* when *E. coli* was used as the host organism where not similar to those obtained when the lichenicidin natural producer was employed. Therefore, the same tests were attempted using *B. licheniformis* I89 strain.

2.2.4 *licR* deletion in *B. licheniformis* I89

Considering the differences of the regulatory mechanisms between Gram positive and Gram negative organisms, *licR* was deleted in the original lichenicidin producer. To achieve this, a shuttle vector (*Bacillus* and *E. coli*) containing an apramycin resistance cassette flanked by approximately 30 bp of *licR* 5' and 3'-ends, was constructed. The plasmid pKSV7 that encodes the resistance to ampicilin in *E. coli* and includes a replication origin that is sensitive to temperature in *Bacillus* (propagation temperature: 30^{o}C; non-replication temperature: 42^{o}C) was used. The shuttle vector constructed was pK*licR*:Apra and it was used for all the transformations performed.

The transformation of *B. licheniformis* is a difficult step regarding the genetic manipulation of this species (Rey, *et al.*, 2004). Thus, several procedures to obtain *B. licheniformis* I89 transformants where attempted in the present study, including transconjugation, electroporation and protoplast transformation (see section 2.4.6). The same plasmid was used in all the different procedures but on the electroporation protocol the solution containing this vector was previously desalted, as salts can interfere with the electric pulse. The *B. licheniformis* MW3 strain was used as a control. In this strain, the genes encoding type I restriction enzymes were deleted, and the transformation efficiency rates were increased (Hoffmann, *et al.*, 2010).

Despite all the protocols tested, it was not possible to obtain a *B. licheniformis* I89 transformant. Consequently, it was not possible to investigate the influence of *licR* in the lichenicidin biosynthesis in the natural producer.

2.3 Conclusions

LicR has homology with several regulatory proteins, mainly those of the HTH_XRE superfamily, which are known to have regulatory functions in many microorganisms. Taking that in account and considering the fact that the first knockout in *E. coli* did not produce Bliα, it was assumed that LicR was a regulatory protein, controlling Bliα biosynthesis. However, herein, it was shown that inhibition of activity was due to an insertion within the *licM1* gene, leading to an incorrect processing of the final α-peptide. Thus, in this study it was found that the absence of LicR does not abolish Bliα production in *E. coli*. Also, the bioactivity results suggested that the production levels were also not affected. Contrarily to what was observed in the bioassay, spectrometry analysis indicated that in BLic5ΔR strain lichenicidin yields are higher when compared with the control BLic5 strain.

Though, considering that the regulation mechanisms of *E. coli* (Gram negative) are significantly different from those of the original producer *B. licheniformis* I89 (Gram positive), the same study was attempted in the original lichenicidin producer strain. Despite the several efforts, it was not possible to transform *B. licheniformis* I89 strain. Consequently, *licR* knockout strain could not be obtained so far.

2.4 Experimental Procedures

2.4.1 Bacterial strains and cultivation media

The characteristics of the *E. coli* strains containing the lichenicidin cluster and used in this section are presented in Table 1. These strains were maintained in Luria-Bertani agar (LA; Merck) plates or grown in Luria-Bertani broth (LB; Merck) at the appropriated temperature. Liquid cultures were performed using medium M containing 10 g/L of NaCl, 10 g/L of tryptone, 5 g/L of yeast extract, 10 g/L of KH_2PO_4, with a final pH of 6.5, adjusted with NaOH (Mendo, *et al.*, 2004). *B. licheniformis* I89 was first isolated from a hot spring in Azores island (Mendo, *et al.*, 2000). *Micrococcus luteus* ATCC 9341 was used as the indicator strain in the bioassay to evaluate lichenicidin production. These two Gram positive strains were maintained routinely in tryptic soy agar (TSA; Merck).

Table 1 – Description of the *E. coli* strains used in this section.

Strain	Description	Phenotype	Reference
BLic5	*E. coli* BL21-Gold(DE3) containing the pLic5 fosmid (entire *lic* biosynthetic cluster)	Clo^R	(Caetano, *et al.*, 2011)
BLic5ΔR	*E. coli* BL21-Gold(DE3) containing the pLic5ΔR fosmid (pLic5 with *licR* gene deleted)	Clo^R	
pKD20/pLic5	*E. coli* BW25113 Containing the pKD20 (oriTS) plasmid and the pLic5 fosmid	Amp^R Clo^R	
S17-1	*E. coli* S17-1	–	(Richhardt, *et al.*, 2010)
ET12567	*E. coli* ET12567 containing the pUZ8002 plasmid	Kan^R Clo^R	(Macneil, *et al.*, 1992)
S17pK*licR*:apra	*E. coli* S17-1 transformed with pK*licR*:apra	Clo^R Amp^R $Apra^R$	This study
ETpK*licR*:apra	*E. coli* ET12567 transformed with pK*licR*:apra	Kan^R Amp^R $Apra^R$	This study

2.4.2 Total RNA extraction

Total RNA from *E. coli* BLic5 and BLic5ΔR strains was purified using the Trizol Max Bacterial Isolation Kit (Invitrogen). The procedure was divided in 3 steps (sample homogenization, phase separation and precipitation of RNA), followed by DNase treatment using Turbo DNA-free kit (Ambion).

2.4.2.1 Sample homogenization

The bacterial strains were cultivated in medium M containing 12.5 µl/mL of Clo with aeration (180 rpm) at 37 ^oC, until an OD_{600nm} of 0.4-0.6. 1.5 mL of this culture was transferred to a pre-chilled microcentrifuge tube and centrifuged at 6000 xg for 5 min at

4 ^{o}C. The supernatant was discarded, the cell pellet resuspended in 200 µl of preheated (95 ^{o}C) Max Bacterial Enhancement Reagent and incubated at 95^{o}C for 4 min. 1 mL of TRIzol® Reagent was added to the lysate and the mixture was incubated at room temperature for 5 min.

2.4.2.2 Phase separation

To the previously obtained lysate 200 µL of cold chloroform were added and the mixture was vigorously shaken by hand for 15 s, incubated at room temperature for 3 min, and then centrifuged at 12 000 xg for 15 min at 4 ^{o}C. After centrifugation, three phases were formed: the lower red phenol-chloroform phase, an interphase and a colorless aqueous phase containing RNA (approximately 400 µl).

2.4.2.3 RNA precipitation

The upper phase containing the RNA was transferred to a new tube, 500 µL of cold isopropanol was added and incubated at room temperature for 10 min, to precipitate RNA. The mixture was centrifuged at 15 000 xg for 10 min at 4 ^{o}C and the supernatant carefully removed. The pellet was washed with 1 mL of 75% ethanol and centrifuged at 7500 xg for 5 min at 4 ^{o}C. Finally, the pelleted RNA was air-dried and resuspended in 50 µL of RNase-free water, followed by incubation for 10 min at 60 ^{o}C.

2.4.2.4 DNase treatment

The contamination of the extracted total RNA with DNA was avoided by treatment with DNase using the Turbo DNA-free kit (Ambion), according with the manufacturer's instructions. Briefly, 5 µL of Turbo DNase buffer and 1 µL Turbo DNase (2U/µl) was added to 50 µL of total RNA. The reaction was carefully mixed and incubated at 37 ^{o}C for 45 min. After incubation, 5.5 µL of DNase Inactivation Reagent was added and the mixture was incubated at room temperature for 2 min. Finally, the reaction was centrifuged at 10 000 xg for 1.5 min and the supernatant containing the RNA was transferred to a new microcentrifuge tube and stored at -80 ^{o}C.

2.4.2.5 Analysis of RNA integrity and concentration

In order to check for RNA integrity, 2 µL of RNA solution were run in an electrophoresis gel 1% agarose. To perform this, electrophoresis new buffer was used.

RNA concentration was determined using Qubit fluorimeter using Quant-iT^{TM} RNA reagents according to manufacturer's instructions, as described in Appendix 11.

2.4.3 Amplification of *licA1* and *licM1* genes

Despite the previous described verification of DNA contamination, a more specific test was performed, to check for the amplification of the target genes, *licA1* and *licM1*. For that, both genes were amplified using the total RNA extracted from both BLic5 and BLic5ΔR strains. Colonies of those strains and *B. licheniformis* I89 were used as positive controls. The primers used for those amplifications are listed on Table 2:

Table 2 – List of primers used to amplify *licA1* and *licM1* and respective sequences and annealing temperatures. The expected size of each amplicon and the extension time for each target gene are also indicated.

Primer name	Primer sequence (5'→3')	Annealing temperature (^{o}C)	Expected amplicon (bp)	Extension time
Comp_licA1 Fw	AGGTGGGATCCATGTCAAAAAAGGAAATG	50	250	45 s
Comp_licA1 Rv	CCCGCCTCGAGAACTTAGTTACAGCTTGGC			
Comp_licM1 Fw	AGGTCGGATCCATGAATGAAAAATCC	52	3181	3 min
Comp_licM1 Rv	CATAGATTCTCGAGTTAAAACACGTTTTC			

The amplification reaction was performed with Taq DNA polymerase (Promega) as described in Appendix 8 using the annealing temperatures indicated in Table 2. PCR products were separated by electrophoresis 1% agarose gel to check for possible contaminations on total RNA reactions.

2.4.4 Production of *licR* knockout mutant

2.4.4.1 Amplification of the disruption cassette

In order to perform the new *licR* knockout mutant in the pLic5 fosmid, an apramycin disruption cassette was amplified using primers binding to the flanking regions of the *licR* gene.

The plasmid pIJ733 was used as template and was extracted as described in Appendix 6**.** The amplification reaction containing 50 ng of template DNA, 0.5 µL of dNTP's (100 mM), 10 µL of Herculase buffer (5X), 0.5 µL of each primer (100 pmol/ µL), 2 µL of DMSO and 1 µL of Herculase II enzyme (5U/ µL), in a final volume of 50 µL. The primers used are listed on Table 3:

Table 3 – Primers used to amplify the disruption cassette *licR*:Apra from pIJ733

Primer	Primer sequence (5'→3')
lanR_Fw	TTTTTGTTATAAACTCTTTACAATGTGTAAAAAACATTGGCTAGCTGTAGGCTGGAGCTGCTTC
lanR_Rv	TCCTTCTCAAATAACGCGGCAATGCGAAACCCCATTAACGCTAGCATTCCGGGGATCCGTCGACC

The amplification program was as follows: 94 ºC for 2 min, 10 cycles with denaturation at 94 ºC for 45 sec, annealing at 50 ºC for 45 sec and extension at 72 ºC for 90 sec, 15 cycles with denaturation at 94 ºC for 45 sec, annealing at 55 ºC for 45 sec and extension at 72 ºC for 90 sec and a final extension at 72 ºC for 5 min.

2.4.4.2 Transformation of *E. coli* BW25113/pKD20/pLic5 with the disruption cassette

The *licR* disruption cassette was used to transform *E. coli* BW25113/pKD20 cells, containing the pLic5 fosmid. The procedure was performed as follows: a pre-culture of this strain was prepared in LB medium containing 100 μg/mL of Amp and 12.5 μg/mL of Clo antibiotics and it was growth at 30 ºC. 100 μL of the culture was used to inoculate 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). The cells were grown at 30 ºC at 160 rpm until an OD_{600} of approximately 0.4 (between 3 to 4 hours). The cells were collected by centrifugation at 6000 xg for 5 min at 4 ºC and washed with 10 mL of ice cold 10 % glycerol. This procedure was repeated once and the cells were finally resuspended on 100 μL of the same solution. For transformation, 50 μL of the prepared cells were mixed with 100-150 ng of the *licR* disruption cassette. The cells were subject to electroporation and the transformants were selected on LA plates containing 50 μg/mL of Apra and 12.5 μg/mL of Clo, grown at 37 ºC. The substitution *licR* gene by the $Apra^R$ cassette was confirmed by colony PCR.

2.4.4.3 Elimination of $Apra^R$ cassette

One positive clone was selected and grown overnight at 37 ºC in LB containing 50 μg/mL of Apra and 12.5 μg/mL of Clo, in order to extract the pLic5ΔR:Apra fosmid. The fosmid was extracted by alkaline lysis as described in Appendix 7. The fosmid was disgested with the restriction enzyme *Bmt*I (New England Biolabs) 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 DNA was precipitated with 1/10 vol of potassium acetate (3 M, pH 5.5) and 0.6 volume of isopropanol. The mixture was incubated at room temperature for 15 min and centrifuged at 4 ºC, 12000 xg for other 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. 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* BL21-Gold(DE3) cells. The transformants were selected on LB agar plates containing 12.5 µg/mL of Clo. The obtained colonies were further cultured on plates containing 12.5 µg/mL of Clo and 50 µg/mL of Apra. The clones presenting the phenotype $Clo^{R}Apra^{S}$ were selected as those containing the *licR* gene deletion without the $Apra^{R}$ cassette. The absence of this cassette was further confirmed by colony-PCR. The integrity of *licM1* gene was also confirmed by colony-PCR as described in section 2.4.3.

2.4.5 Construction of plasmid for *licR* disruption in *Bacillus*

2.4.5.1 Insertion of *licR*:Apra cassette into pKSV7 vector

In order to obtain a *Bacillus licheniformis* I89 *licR* mutant, it was necessary to construct a plasmid containing a *licR* disruption cassette that was able to replicate in *Bacillus*. To achieve this, the plasmid pKSV7 was used as vector (Li & Kathariou, 2003). This plasmid contains an origin of replication for *Bacillus* sensitive to the temperature (permissive temperature 30 ºC), an *E. coli* origin of replication, a *cat* gene conferring resistance to chloramphenicol and the pUC19 multiple cloning site. This plasmid was extracted using the QIAprep Spin MiniPrep Kit (QIAGEN), according with manufacturer's instructions. Approximately 600 ng of pKSV7 vector was digested with 10 U of *Sma*I restriction enzyme (Fermentas) in a reaction with a final volume of 40 µL, containing 1X Tango buffer. The reaction was incubated at 30 ºC for 1 hour. *Sma*I digestion will generate blunt ends, meaning that the *licR*:$Apra^{R}$ disruption cassette amplified in section 2.4.4.1 can be directly used to perform a blunt-end ligation.

After digestion, the plasmid was purified using the JETquick Purification kit (Genomed) as described in Appendix 10 and its concentration was determined using Qubit® (Appendix 11). The ligation reaction was performed in a final volume of 20 µL, containing: 50 ng of *Sma*I digested pKSV7, 250 ng of *licR*:Apra^R cassette, 1x of T4 DNA ligase buffer, 5U of T4 DNA ligase and 2 µL of 50 % PEG 4000 solution. The reaction was incubated at 22 ^oC for 1 h and then stored at -20^oC until further use.

2.4.5.2 Transformation

To ensure the integrity and functionality of the pK*licR*:Apra, a subcloning procedure was carried out using chemically competent *E. coli* DH5α cells. 5 µL of the ligation were used for transformation procedure and the transformation was performed by heat shock as described in the Appendix 4. Transformants were selected overnight at 37 ^oC on LA plates containing 100 µg/mL of Amp and 50 µg/mL of Apra.

Positive clones were selected using colony-PCR with the appropriate primers using the protocol described in Appendix 8 using lanR primers (Table 3). One of the positive clones was isolated in a new LA plate containing the same selective markers and used to extract the pK*licR*:Apra plasmid with the alkaline lysis procedure described in Appendix 7.

After extraction, the plasmid was treated with RNase at a final concentration of 2 mg/mL during 1 hour at 37 ^oC. Then, 1 volume of Phenol/CIA was added to remove proteins and shaken. The solution was centrifuged in a top-table centrifuge at top speed for 5 min and the upper organic phase was collected to a clean 1.5 mL microcentrifuge tube. 1/10 volume of NaAc and 0.6 volume of isopropanol were added and the suspension was left for 10 min on the table to let precipitation to occur. A new centrifugation was performed at 4 ^oC, top speed for 15 min. the supernatant was discarded and the plasmid DNA was resuspended in 500 µL of 70 % ethanol. The suspension was centrifuged as mentioned and the supernatant was discarded. The pellet was air-dried to remove residual ethanol and then resuspended in 200 µL of distilled water.

2.4.6 *B. licheniformis* transformation

In order to produce a knockout strain of *B. licheniformis* diverse protocols described for *Bacillus* transformation were tested and improved, including transconjugation, electroporation and protoplasts transformation.

2.4.6.1 Transconjugation using *E. coli* strains

The transconjugation protocol applied in this study was adapted from Richhardt *et al* (Richhardt, *et al.*, 2010). The procedure was tested using two different donor strains: the *E. coli* S17-1 and the *E. coli* ET12567. The first strain is able to methylate DNA and the other is not able to methylate it. This could allow to understand if DNA methylation could influence the intake of pDNA by I89 strain. Thus, chemically competent cells were prepared for both *E. coli* strains and transformed with pK*licR*:Apra using heat shock protocol (Appendix 4).

In general terms, *B. licheniformis* and the two *E. coli* strains containing the pK*licR*:Apra plasmid were inoculated in 5 mL of LB medium with the appropriate selective markers (see Table 1). The cultures were grown overnight at 37 ^{o}C. Then, 50 mL of LB were inoculated with 1 mL of Bacillus culture and 50 mL of LB with the appropriate antibiotics were inoculated with 1 ml of each one of the overnight cultures and allowed to grow until the OD_{600} reached 0.6-0.8. Each culture was centrifuged at 4 ^{o}C for 15 min at 3200 xg and the cell pellets resuspended in 15 mL of holding buffer (12.5 mM KH_2PO_4, 12.5 mM K_2HPO_4, 1 mM $MgSO_4$, pH 7.2). These two steps were repeated twice and the last resuspension was performed in 30 ml of holding buffer. At this stage, the cells were prepared for transconjugation by direct contact and also using filter matting. Also, the influence of *B. licheniformis* I89 incubation at 49 ^{o}C before the transconjugation procedure described by Richhardt, *et al*. (2010) was tested.

Briefly, 10 mL of *B. licheniformis* I89 culture (either with or without 49 ºC treatment) was mixed with 5 mL of each one of the *E. coli* donor strain (2:1). Afterwards, two distinct approaches were adopted:

a) Direct contact: 1 mL of the bacterial mixture was spread in LA plates in duplicates and one plate was incubated at 30 ºC and other plate at 37 ºC for 24 h. Following this, each plate was washed with 1 mL of LB medium.

b) Filter matting: 3 mL of the bacterial mixture was filtered with 0.45 µm nitrocellulose filters. This was performed in duplicates and each one of the filters was placed on a LA plate with the cells forming the top layer. One plate was incubated at 30 ºC and other plate at 37 ºC for 24 h. Following this, each filter was transferred to a 2 mL microcentrifuge tube containing 900 µL of LB medium and mixed.

For both procedures, the volume of bacterial suspension obtained was divided in two 1.5 mL microcentrifuge tubes (approximately 450 µL in each). One of the tubes was treated at 80 ^{o}C for 20 min, in order to select *B. licheniformis* I89 spores. After this,

both tubes were centrifuged at 6000 xg for 2 min and the most of the supernatant was discarded. The resulting pellet was resuspended in the remaining supernatant (approximately 100 µL) and plated in LB agar plates containing the appropriate antibiotics (12.5 µg/mL of Clo and 50 µg/mL of Apra). All the plates were incubated for 24 h at 30 ºC.

2.4.6.2 Electroporation

Electroporation is a simple and rather efficient method to transform bacterial strains. However, it is known that *B. licheniformis* strains are among the most difficult transformable strains. Thus, electroporation was tested to transform *B. licheniformis* I89 strain, using a protocol adapted from Tamagnini, *et al* (Tamagnini, *et al.*, 2008).

A pre-culture of I89 was performed using 5 mL of LB containing 0.5 M of sorbitol and grown at 37 ^{o}C with aeration (180 rpm), overnight. The culture was diluted 20-fold in the same medium and grown at 37 ^{o}C with 250 rpm until an OD_{600nm} of 1-1.1 was reached. Afterwards, it was centrifuged at 4 ^{o}C at 5000 xg for 5 min and the resulting pellets were washed twice with ice-cold electroporation solution (0.5 M sorbitol, 0.5 M mannitol and 10% glycerol). Finally, the pellet was resuspended in 1/40 volume of the same solution. For electroporation, 60 µL of the prepared electrocompetent cells were mixed with 50 ng of pK*licR*:Apra. The pK*licR*:Apra vector was previously desalted using a desalting membrane (Millipore) placed at the surface of a plate containing distilled water, for 15 min and transferred to a new tube. The electroporation was performed using 1 mm gap electroporation cuvettes (Bio-Rad) and a single electric pulse was given at 2.1 kV in the MicroPulser Electroporator (Bio-Rad). After pulse, 1 mL of LB medium containing 0.5 M of sorbitol and 0.38 M of mannitol was immediately added and the suspension was incubated at 30 ^{o}C at 150 rpm for 3 h in a 15 ml tube. The culture was finally plated onto LB agar medium with the appropriate selective markers (50 µg/mL of Apra) and incubated for 3 days at 30 ^{o}C. The plates were routinely monitored.

2.4.6.3 Transformation of protoplasts

Transformation of protocol is one of the most used procedures to transform *Bacillus* and other hardly transformable strains. The protocol applied in this study, was adapted from Horn and Waschkau, *et al* (Horn, 1990, Waschkau, *et al.*, 2008) and included

some modifications kindly suggested by Dr. Claudia Borgmeier (AK Prof. Dr. F. Meinhardt, WWU Münster Institut für molekulare Mikrobiologie und Biotechnologie).

30 mL of #416 medium were inoculated with a single colony using a 250 mL flask and grown overnight at 37 ^{o}C and 250 rpm. The overnight culture was diluted to an to an OD_{600nm} of 0.1 in 100 mL of #416 medium and incubated at 37 ^{o}C, 250 rpm until the 0.4-0.5 in the following ones. The culture was then transferred to a 50 mL sterile falcon tube and centrifuged at 4 ^{o}C at maximum rotation speed for 15 min. The pellet was resuspended in 5 mL of SMMP supplemented with 130 µL of freshly prepared lysozyme. The mixture was incubated at 37 ^{o}C with 90 rpm during approximately 30 min. 20 mL of SMMP were added and gently mixed, followed by a centrifugation at 2200 xg as mentioned for 10 min. The remaining pellet was resuspended in 5 ml of SMMP followed by a short heat step at 65 ^{o}C for 5 min to inactivate restrictases. The suspension was centrifuged at 1400 xg for 8 min at room temperature and 1 mL of SMMP/BSA were added to the cell pellet.

25 µL of pK*licR*:Apra DNA (100 ng/µL) was mixed with 25 µL of 2x SMM in a sterile 1.5 microcentrifuge tube. 500 µL of the prepared protoplasts was transferred to the tube containing the pDNA. 1.6 mL of 40 % PEG 8000 (prepared with 1x SMM) was placed in a 50 mL falcon tube and the mixture of protoplasts-plasmid was then transferred to this tube. The solution was gently shaken during 2 min at room temperature and of 5 mL of SMMP+ was added. The protoplasts were recovered by centrifugation at 8 ^{o}C at 500 xg during 8 min and finally resuspended in 1 mL of SMMP+. The suspension was incubated during 2h at 37 ^{o}C, 130 rpm standing angled.

After incubation, the protoplasts were plated on DM3 agar supplemented with the appropriate selective marker (12.5 µg/mL of Clo and 50 µg/mL of Apra) and in DM3 without antibiotics in order to estimate the number of regenerated protoplasts. Also, serial dilutions were performed (10^{-2}/10^{-5}) and plated on LB agar in order to obtain the number of non-protoplasted cells. Air bubbles must be avoided when doing the plates. The plates were incubated during 2-5 days at 37 ^{o}C.

Solutions:

#416 medium: per 1 l – 20 g of peptone, 10 g of yeast extract, 10 g NaCl, 100 mL 2 M sucrose (freshly added).

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

2x SMM: 1 M sucrose, 0.04 M sodium maleate and 0.04 M MgCl2.6H2O. Sterilize in the autoclave for 10 min.

0.2 N sodium maleate: per 250 ml – 5.8 g maleic acid in 50 mL of 1N NaOH. Add sterile water until the desired volume.

4xPAB: per 1 l – 6 g beef extract, 6 g yeast extract, 20 g peptone, 4 g dextrose, 14 g NaCl, 14.72 g K_2HPO_4, 5.28g KH_2PO_4

SMMP+ medium: 100 mL of SMMP with 0.2 mL of 20 % BSA (filter sterilized)

Lysozyme solution: 10 mg/mL in 1x SMMP (filter sterilized; freshly prepared)

40 % PEG (w/v): 10 g PEG 8000 in 25 mL of 1x SMM. Sterilize in the autoclave for 10 min.

DM3 regeneration agar/succinate based regeneration agar: 200 mL of 4 % agar (Cf=0.8 %), 500 mL of 1 M sodium succinate (acid succinic) pH 7.3 (Cf=0.5 M), 100 mL 5 % casaminoacids (Cf=0.5 %), 50 mL 10 % yeast extract (Cf=0.5 %), 100 mL 3.5 % K_2HPO_4, 1.5 % KH_2PO_4 (Cf=0.35 %, 0.15 %), 15 mL 40 % glucose (Cf=0.6 %), 20 mL 1 M $MgCl_2$ (Cf=0.02 M), 10 mL of sterilized dH_2O, 5 mL of 20 % BSA (added to the mixture at approximately 55^{o}C; Cf=0.1 %).

2.4.7 Bioassay

2.4.7.1 Preparation of extracts

Bacterial strains were cultivated in 5 mL of medium M supplemented with the appropriated selective marker, at 37 ^{o}C, 180 rpm and overnight. 300 µl of this culture was used to inoculate 30 mL of medium M and incubated for 24 h at 37 ^{o}C, 180 rpm. This procedure was performed in triplicates for each strain. Afterwards, 5 mL of 1-butanol (Merck) were added to 20 mL of the bacterial culture and shaken for 1 h. The mixture was centrifuged for 1 min at 6000 xg. 2 ml of the organic upper phase were collected and divided into two 1.5 mL microcentrifuge tubes. The organic solvent was evaporated at 50 ^{o}C for 3 hours using a SpeedVac evaporator (Labconco). For each replica, one pellet was stored at -80 ºC and sent for HPLC-ESI-MS/MS analysis. The other pellet was dissolved in 500 µL of 70% ACN:water and used for bioactivity quantification. For each replica, one tube was used to perform bioassays and the other one was sent to HPLC-ESI-MS/MS.

2.4.7.2 Quantification by bioassay

Twofold serial dilutions of the extracts were performed for each replica and 50 µL of each dilution were dispensed into wells previously made in the bioassay agar plates, containing the indicator strain *M. luteus*. After overnight incubation at 37 ^{o}C the inhibition halos were analyzed.

The peptide activity was expressed as arbitrary units (AU). The arbitrary units per milliliter (AU/ml) were calculated using the reciprocal of the last dilution that gave a distinct zone of inhibition multiplied by the conversion factor (Ryan, *et al.*, 1996).

CHAPTER III

NEW EXPRESSION SYSTEM FOR BLIα AND BLIβ PRODUCTION IN *E. COLI*

3.1 Background

One of the major advantages of using the *E. coli* system for heterologous expression is that this Gram negative bacteria is very amenable to genetic manipulation.

The lichenicidin heterologous expression system in *E. coli* was firstly used to produce both α and β peptides in the same strain. In this system, the entire lichenicidin gene cluster was located on a fosmid, where the all the genes expressed are regulated by *B. licheniformis* determinants. However the production of both peptides simultaneously is not advantageous concerning downstream processing; so it was attempted to produce strains capable of synthesizing each peptide independently. For that, two strategies have already been developed:

– Deletion of *licA1* (to produce only Bliβ) or *licA2* (to produce only Bliα) gene from the fosmid pLic5 (Caetano, *et al.*, 2011). In these cases, the biosynthesis is still controlled by *B. licheniformis* determinants.

– Deletion of *licA1* and *licA2* from pLic5 fosmid and transcomplementation with the respective gene into pET-24a(+) or pUC19a vectors (Caetano, *et al.*, 2011, Cruzeiro, 2012). In these cases only the expression of the structural genes is under the control of *E. coli* genetic determinants. The major advantage of this system is the easier manipulation of the structural gene allowing the attainment of variants of those genes.

All of these systems involve the presence of the complete *lic* biosynthetic cluster inserted into a fosmid (approximately 25 Kb). Due to its high molecular weight, this structure can be instable. Also, the presence of the complete cluster can require more energy, so it could be advantageous to have two different strains producing each single peptide, since less energy would be necessary to express the genes involved and possibly making the process faster and more efficient. Thus, the production of Bliα and Bliβ separately in *E. coli* was attempted, using a construct of lower molecular weight. To achieve this, it was decided to clone only the genes necessary for Bliα (*licA1*, *licM1* and *licT*) or Bliβ (*licA2*, *licM2*, *licT* and *licP*) production into a plasmid. The plasmids were inserted into *E. coli* BL21-Gold(DE3) host and the production of the peptides was investigated by colony bioassay using *E. coli* strains producing the complementary peptide. Moreover, the levels of lichenicidin production for each system available were compared.

3.2 Results and Discussion

3.2.1 Construction of p*licA1M1T* and p*licA2M2TP*

To produce only Bliα, the essential genes for its biosynthesis (*licA1*, *licM1* and *licT*) were cloned into pET-24a(+) as explained in section 3.5 to originate the plasmid p*licA1M1T*. A similar approach was carried out for Bliβ production. In this case, the *licA2*, *licM2*, *licT* and *licP* genes were inserted in the same plasmid to produce the p*licA2M2TP* plasmid, as explained in section 3.5.2. Both plasmids were transformed in *E. coli* BL21-Gold(DE3) cells, producing BpA1M1T (Bliα) and BpA2M2TP (Bliβ) strains.

In order to understand if the lichenicidin peptides were being produced by these new expression systems, a colony bioassay was performed where, BLic5ΔA1 (Bliβ) and BLic5ΔA2 (Bliα) were used as complementary producer strains (Figure 20).

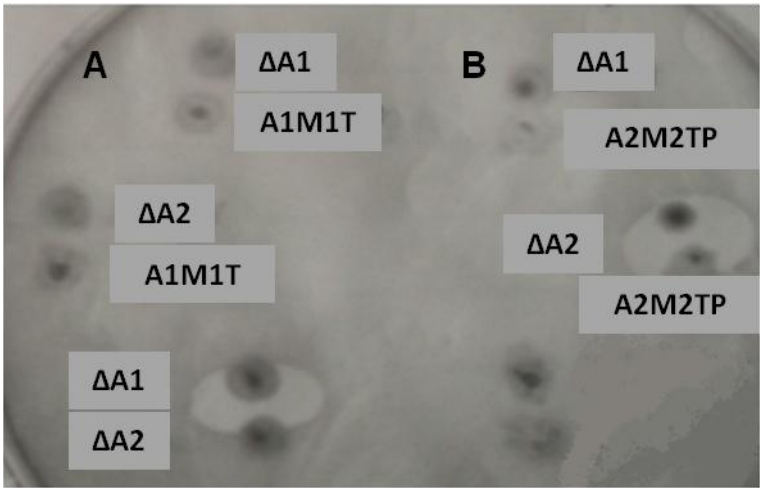

Figure 20 - (A) Bioassay of the BpA1M1T (A1M1T) strain with the complementary producer BLic5ΔA1 (ΔA1). The strain BLic5ΔA1 (ΔA2), producing Bliα was used as a negative control. (B) Bioassay of the BpA2M2TP (A2M2T) strain with the complementary producer BLic5ΔA2. BpA2M2TP presented activity when acting synergistically with BLic5ΔA2 but not with BLic5ΔA1. (A,B) BLic5ΔA1 and BLic5ΔA2 were bioassayed side-by-side, as positive control.

As shown in Figure 20, the strain containing the p*licA1M1T* showed no synergy activity with the BLic5ΔA1 strain against *M. luteus*. This suggested that Bliα was not produced. Despite several attempts using this strategy, it was not possible to obtain a Bliα-producer strain. One possible explanation relies on the fact that the α-peptide could possivly present some activity against the host cell due to its mode of action. In fact, studies show that the α-peptide is the first to attache to the cell membrane, binding preferentially to lipid II, but also to lipid I, thereby preventing peptidoglycan biosynthesis and working as doking site for the β peptide (Oman & van der Donk, 2009). Thus, only cells containing possibly interrupted genes will survive, once the

peptide is not correctly produced avoiding the attachment to the producer cell membrane. Another explanation could be related with the expression of the immunity genes that are absent in this strain. Some studies reference that the over expression of the immunity genes lead to enhanced lantibiotic production (Koponen, *et al.*, 2004, Hu, *et al.*, 2010). This would imply that if the host contains improved protection against the peptides, it could increase their production levels. This hypothesis was not considered for the BpA1M1T strain construction, since it was previously described that Bliα was produced in the absence of the immunity genes, *licFGEHI*, in *E. coli* (Caetano, *et al.*, 2011).

The strain containing the p*licA2M2TP* plasmid presented bioactivity when working synergistically with BLic5ΔA2 (Bliα) (Figure 20). This result showed that a fully active Bliβ peptide was being produced by BpA2M2TP strain. In this strain, the immunity genes were also not present in this strain ant still, the Bliβ peptide was produced. Since it was possible to obtain this strain, a comparison of the Bliβ production levels by the expression systems available was performed and is presented in the following section.

3.3 Comparison of Bliβ production levels

To compare the Bliβ production levels between the available systems the *E. coli* strains were grown in liquid media and the peptides were extracted from the culture. These strains included *E. coli* BpA2M2TP, *E. coli* BLic5ΔA1 (pLic5ΔA1), *E. coli* BLic5ΔA1ΔA2+p*licA2* (pET-24a(+) and *licA2*) and *E. coli* BLic5ΔA1ΔA2+pUC*A2* (pUC19a and *licA2*) (Table 4, section 3.5.1). After extraction, the same sample of each replicate was divided into two tubes. One was used to perform a bioassay and the other was analyzed by HPLC-ESI-MS. Both strategies were carried out to compare the production levels of Bliβ.

Regarding the quantification by bioassay, serial dilutions of each replica were performed and tested against *M. luteus*. The value of the last well showing inhibition was considered to calculate the arbitrary units per milliliter (AU/mL).

This value was used to compare the bioactivity of the various samples. Thus, higher AU/mL values will indicate the presence of higher amounts of the Bliβ peptide.

The results (Figure 21) showed that bioactivity was similar in all the tested strains. Nevertheless, the extract obtained from BpA2M2TP strain seems to have a slightly decreased activity. This suggests that the amounts of Bliβ peptide produced by this

strain should be lower than those of the other strains. One possible explanation could be the need for other genes of the gene cluster that are absent only in this strain.

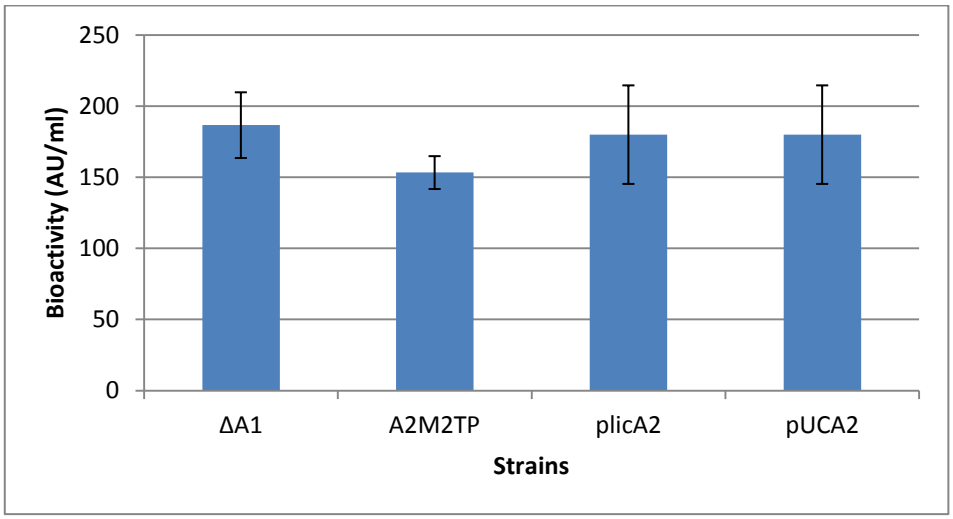

Figure 21 – Quantification of Bliβ production by bioassay against *M. luteus*. The AU/mL corresponds to the last well of the successive double dilutions that showed activity. ΔA1 – BLic5ΔA1; A2M2TP – BpA2M2TP; pETA2 – BLic5ΔA1ΔA2+p*licA2*; pUCA2 – BLic5ΔA1ΔA2+pUC*licA2*.

In order to have a more accurate outcome, these results obtained by bioassay should always be compared with those obtained with quantification data retrieved from HPLC-ESI-MS/MS analysis (Figure 22).

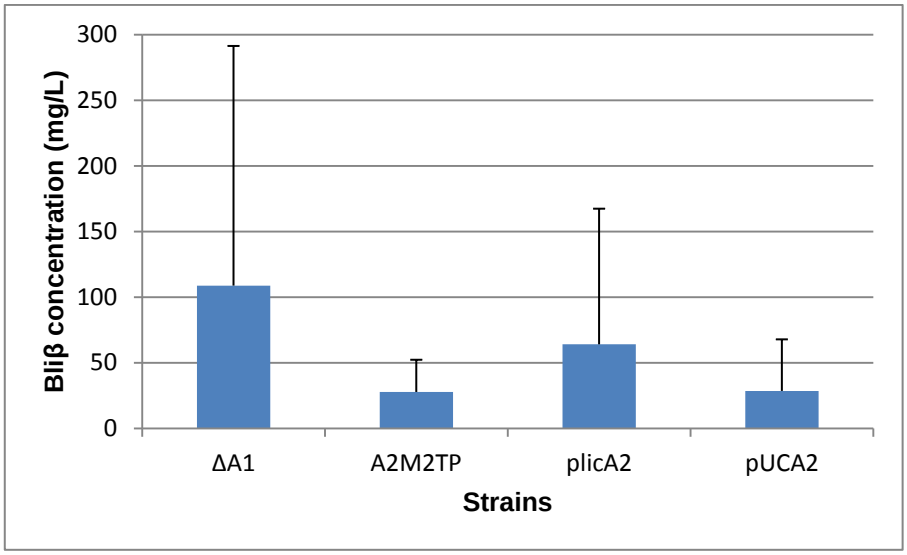

Figure 22 – Quantification of Bliβ production by HPLC-ESI-MS/MS. ΔA1 – BLic5ΔA1; A2M2TP – BpA2M2TP; pETA2 – BLic5ΔA1ΔA2+p*licA2*; pUCA2 – BLic5ΔA1ΔA2+pUC*licA2*.

The MS analysis evidenced the lower Bliβ production by BpA2M2TP strain and the higher yield by BLic5ΔA1 strain. The major difference observed between bioactivity and MS results was with BLic5ΔA1ΔA2+pUC*licA2* strain. This can be due to the fact that the bioassay method is not very precise and probably the production differences are

quite small to induce major variations in the inhibition areas. Thus, lower variations in the production levels would be difficult to detect when using a phenotypic method. The mass spectrometry analysis is much more precise and reliable. Moreover it was observed that the standard deviations obtained for the samples analyzed by MS were high. This indicates the discrepancy of the production levels detected between biological replicas. Therefore in future studies, the analysis of a higher number of replicates would be suggested in order to improve the accuracy of the results.

3.4 Conclusion

Considering all of the results herein presented, it seem reasonable to state that it is possible to produce each lichenicidin peptide independently and under the control of the *E. coli* promoter, without needing the original regulatory proteins to control the biosynthesis. This is supported by the fact that Bliβ was produced by BpA2M2TP strain. However, Bliα biosynthesis using the BpA1M1T strain it could not be achieved. Thus, further investigation is required in order to understand why the host was not able to cope with the vector containing the essential genes to Bliα production and how this problem could be overpassed. Moreover, MS results suggest that the new system developed (BplicA2M2TP) was not beneficial for Bliβ production. Therefore, additional studies should be performed to clarify if such system can be improved.

3.5 Experimental Procedures

3.5.1 Bacterial strains and cultivation media

The characteristics of the *E. coli* strains containing the lichenicidin cluster and used in this section are presented in Table 4. These strains were maintained in Luria-Bertani agar (LA; Merck) plates or grown in Luria-Bertani broth (LB; Merck) at the appropriated temperature. Liquid cultures were performed using medium M containing 10 g/L of NaCl, 10 g/L of tryptone, 5 g/L of yeast extract, 10 g/L of KH_2PO_4, with a final pH of 6.5, adjusted with NaOH (Mendo, *et al.*, 2004).

Table 4 – Description of the *E. coli* strains used in this section. LBM stands for strain belonging to Laboratory of Molecular Biotechnology.

Strain	Description	Phenotype	Reference
BLic5ΔA1	*E. coli* BL21-Gold(DE3) containing the pLic5ΔA1 fosmid (pLic5 with *licA1* gene deleted)	Clo^R	(Caetano, *et al.*, 2011)
BLic5ΔA2	*E. coli* BL21-Gold(DE3) containing the pLic5ΔA2 fosmid (pLic5 with *licA2* gene deleted)	Clo^R	(Caetano, *et al.*, 2011)
BLic5ΔA1A2	*E. coli* BL21-Gold(DE3) containing the pLic5ΔA1A2 fosmid (pLic5 with *licA1* and *licA2* genes deleted)	Clo^R	LBM
BpA1M1T	*E. coli* BL21-Gold(DE3) containing the p*licA1M1T* plasmid (pET-24a(+) with *licA1*, *licM1* and *licT* genes inserted)	Kan^R Clo^R	This study
BpA2M2TP	*E. coli* BL21-Gold(DE3) containing the p*licA2M2TP* plasmid (pET-24a(+) with *licA2*, *licM2*, *licT* and *licP* genes inserted)	Kan^R Clo^R	This study
BLic5ΔA1ΔA2 + p*licA2*	*E. coli* BL21-Gold(DE3) containing the pLic5ΔA1A2 fosmid (pLic5 with *licA1* and *licA2* genes deleted) and p*licA2* (pET-24a(+) with *licA1* gene)	Kan^R Clo^R	(Cruzeiro, 2012)
BLic5ΔA1ΔA2 + pUC*A2*	*E. coli* BL21-Gold(DE3) containing the pLic5ΔA1A2 fosmid (pLic5 with *licA1* and *licA2* genes deleted) and pUC*A2* (pUC19a with *licA2* gene)	Amp^R Clo^R	(Cruzeiro, 2012)

3.5.2 Construction of p*licA1M1T* and p*licA2M2TP*

3.5.2.1 Amplification of the fragments

The construction of the plasmids p*licA1M1T* and p*licA2M2TP* involved two-step cloning of PCR products. To obtain the p*licA1M1T* plasmid, three different strategies were used and are represented in Figure 23. For p*licA2M2TP* plasmid construction, *licA2M2* was amplified and cloned in pET-24a(+) plasmid between the *Bam*HI and *Not*I restriction sites. The second step involved the insertion of *licTP* amplification in the *Not*I restriction site of p*licA2M2* plasmid.

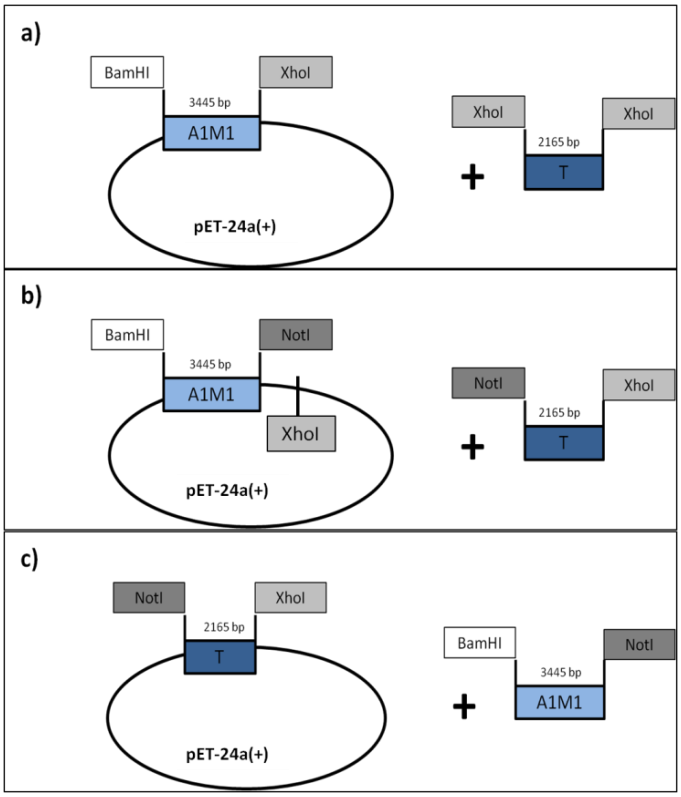

Figure 23 – General plan of experiments to construct plicA1M1T. a) and b) first, *licA1M1* was inserted followed by *licT*; c) insertion of *licT*, followed by *licA1M1*; a) *licT* with same cohesive ends.

The amplification of *licA1M1*, *licA2M2*, *licT* and *licTP* fragments was performed in a 50 µL reaction containing 0.5 µL of dNTPs (25 mM), 10 µL of Herculase II Buffer (5X), 1 µL of DMSO, 1.25 µL of each primer (10 pmol/µL), 100-400 ng of total DNA of *B. licheniformis* I89 and 5 U of Herculase II DNA polymerase. The primers applied are listed in Table 5.

Table 5 - List of primers used to perform the amplifications of *licA1M1*, *licA2M2*, *licT* and *licTP* genes. In bold is represented the recognition site for the restriction enzyme used. The initiation codon is underlined.

Primer	Primer sequence (5'→3')	Restriction Enzyme
Comp_licA1_Fw	AGGTG**GGATCC**ATGTCAAAAAAGGAAATG	*Bam*HI
Comp_licM1_Rv	CATAGATT**CTCGAG**TTAAAACACGTTTTC	*Xho*I
Comp_licM1_Rv_Not	CTAGATT**GCGGCCGC**TTAAAACACGTTTTC	*Not*I
licT_RBS_Xho_Fw	TA**CTCGAG**AGGAGGTATAAGGCATGTTTTTTCATAAGA	*Xho*I
licT_RBS_Not_Fw	TAG**CGGCCG**CAGGAGGTATAAGGCATGTTTTTTCATAAGA	*Not*I
Comp_licT_Rv	GGTGGTGGTG**CTCGAG**TCACATCATCACCTCTGCAGATT	*Xho*I
Comp_licA2_Fw	ATCA**GGATCC**ATGAAAACAATGAAAAATTCAG	*Bam*HI
Comp_licM2_Rv	TAGTG**CGGCCG**CTCACCTGCCCGTCGGAATATC	*Not*I
Comp_licP_Rv	TTTTG**CGGCCG**CTCACTCCTTGTTCATCATTTTC	*Not*I

The amplification program included 95 ºC for 2 min, followed by denaturation at 95 ºC for 20 sec, annealing at specific temperature (Table 6) for 20 sec and extension at 72 ºC for specific time (Table 6). The final extension step was performed at 72 ºC for 3 min.

Table 6 - Annealing temperature and extension time used in the PCR reactions performed to amplify *licA1M1*, *licA2M2*, *licT* and *licTP* genes.

Amplification	Primers	$T_{annealling}$ (ºC)	Extension time (min)
licA1M1	Comp_licA1_Fw Comp_licM1_Rv	54	4
licA1M1	Comp_licA1_Fw Comp_licM1_Rv_Not	54	4
licT	licT_RBS_Xho_Fw Comp_licT_Rv	57	3
licT	licT_RBS_Not_Fw Comp_licT_Rv	57	3
licA2M2	Comp_licA2_Fw Comp_licM2_Rv	58	4
licTP	licT_RBS_Not_Fw Comp_licP_Rv	56	4

3.5.2.2 Digestion

In order to insert the fragments amplified into the chosen vector, and considering the experiments previous listed, a range of digestions were performed to cover all the situations (Table 7). All reactions were carried out in a final volume of 40 µL containing 1000 ng of insert or 700 ng of plasmid, the appropriate enzyme and reaction buffer (Fermentas; Table 8). The digestions were performed at 37 ^{o}C for 1 hour and purified with NZYGelpure kit (NZYtech) according to the manufacturer's instructions (Appendix 10).

Table 7 – Table of digestion reactions performed to all plasmid used and respective fragments.

	licA1M1	***licT***	***licA2M2***	***licTP***
pET-24a(+)	*Bam*HI/*Xho*I *Bam*HI/*Not*I	*Not*I/*Xho*I	*Bam*HI/*Not*I	-
p*licA1M1*	-	*Xho*I *Not*I/*Xho*I	-	-
p*licT*	*Bam*HI/*Not*I	-	-	-
p*licA2M2*	-	-	-	*Not*I

Table 8 – List of buffers and restriction enzymes used in the digestion reactions performed. The double digestions were prepared according with DoubleDigest^{TM} (Fermentas) indications.

***Bam*HI/*Not*I**	***Bam*HI/*Xho*I**	***Not*I/XhoI**	***Xho*I**
Buffer O (1x)	Buffer *Bam*HI (1x)	Buffer O (1x)	Buffer O (1x)
10 U of *Not*I 40 U of *Bam*HI	10 U of *Bam*HI 20 U of *Xho*I	10 U of *Not*I 20 U of *XhoI*	10 U of *Xho*I

Ligation reactions were performed in a total volume of 20 µL containing 50 ng of plasmid DNA, 150 ng of DNA insert, 1X T4 DNA ligase buffer and 1 µL of T4 DNA ligase (Fermentas). All reactions were incubated at 22 ^{o}C for 1 hour on a thermocycler and conserved at -20 ^{o}C until further use.

3.5.2.3 Transformation

The subcloning procedures were carried out with chemically competent *E. coli* DH5α cells, using 5 µL of the ligation for transformation procedure. Once the final p*licA1M1T* and p*licA2M2TP* plasmids were obtained, 2 µL of the plasmid were used to transform chemically competent *E. coli* BL21-Gold(DE3) cells. Transformations were performed by heat shock using chemically competent *E. coli* cells as described in the Appendix 4. Transformants were selected overnight at 37 °C on LA plates containing 50 µg/mL of Kan.

Positive clones were selected using colony-PCR with the appropriate primers using the protocol described in Appendix 8.

3.5.2.4 Screening and Bioassay

The colony-bioassay was performed as described in 2.4.7.

3.5.3 Comparison of Bliβ production levels

3.5.3.1 Preparation of extracts

Bacterial strains were cultured and peptide's extraction was performed as described in 2.4.7.1.

3.5.3.2 Quantification by bioassay

The strains producing exclusively Bliβ peptide do not exhibit antibacterial activity against *M. luteus*. Therefore, in order to measure the bioactivity of these extracts, the Bliα peptide needed to be provided on the agar plates. These agar plates were prepared with the supernatant of an *E. coli* BLic5ΔA2 culture. For this, *E. coli* BLic5ΔA2 was pre-cultured (Medium M supplemented with 12.5 µg/mL of Clo), at 37 °C, 180 rpm, overnight) and 1 mL was used to inoculate 100 mL of medium M. After 24 h at 37 °C, 180 rpm, the culture was centrifuged twice at 12 000 xg for 5 min and the supernatant filtered using a 0.45 µm nitrocellulose filter. 6.25 mL of this supernatant was added to 42.75 mL of medium M containing 1.75 % agar for each plate. After mixing, *M. luteus* was added to a final OD_{600nm} of 0.02 and the plates prepared.

The extracts bioactivity was quantified as previously described in section 2.4.7.2.

CHAPTER IV

RANDOM MUTAGENESIS OF BLIα PEPTIDE

4.1 Background

Lantibiotics present a major advantage over the usual antibiotics in what concerns to bioengineering, since the final peptide is gene encoded and thus, much more amenable to engineering strategies. Those approaches can contribute not only to produce peptides with altered biological, chemical and physical properties but also to the lantibiotics structure-function elucidation (Field, *et al.*, 2010). Indeed, several approaches have been developed during the last years in order to obtain peptides with different characteristics from those of the originally produced. These changed peptides have been produced and studied with two major goals: i) to get deeper insights in structure-activity relationships and ii) to obtain improved variants in terms of activity and/or production (Appleyard, *et al.*, 2009, Field, *et al.*, 2010).

The most common approaches used nowadays are related with mutagenesis techniques, usually random mutagenesis or site-directed mutagenesis (Field, *et al.*, 2010). This last one, can also include the site-saturation mutagenesis, in which it is tried to generate all possible mutations at a specific site (Appleyard, *et al.*, 2009). The site-directed mutagenesis implies a mutation in a specific nucleotide while in random mutagenesis several mutations can be inserted randomly within the gene of interest. All of these methods have already been applied to the lantibiotic' study (Field, *et al.*, 2007, Appleyard, *et al.*, 2009, Field, *et al.*, 2010).

In the present study random mutagenesis was the method chosen. The main advantage of this system is that is possible to obtain a large number of mutants containing the most variable mutations, which might increase the different activities observed. Also, this technique does not require previous knowledge about the gene sequence, once the mutations are inserted randomly. On the contrary, it requires an easy screening method, since sometimes it is not easy to understand which mutation is causing a specific phenotype.

For this approach, *licA1* from the original *B. licheniformis* I89 was used to perform mutagenesis. Mutations are randomly inserted in the selected using a procedure that uses a high frequency of error insertion DNA polymerase; mutants are generated that can differ in a single or many nucleotides or may even include insertions. Then, the mutated PCR products were ligated to the pUC19a vector and introduced into an *E. coli* strain containing the pLic5 fosmid in which licA1 and licA2 were deleted

(BLic5ΔA1ΔA2 strain). With this, it was expected to obtain a number of mutations that produce could interfere with the bioactivity and/or production of Bliα.

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4.2 Results and Discussion

4.2.1 *licA1* library

To produce a library of *licA1* mutants, the original *licA1* gene was submitted to two cycles of amplification with Mutazyme II, in order to increase the number of induced mutations. The resulting PCR product that undergone random mutagenesis, was ligated with pUC19a plasmid and transformed in *E. coli* BLic5ΔA1ΔA2, which includes the whole lichenicidin gene cluster except both structural genes. From this procedure, approximately 3030 clones were picked and tested by colony-bioassay using BLic5ΔA1ΔA2+pUC*licA1* as positive control.

4.2.2 Analysis the library by colony-bioassay

The first screening of the library was performed by colony-bioassay, in order to narrow the number of clones that would be further investigated. The bioassay was performed by replica plating using *M. luteus* as indicator strain. To obtain inhibition areas, the supernatant of the BLic5ΔA1 was incorporated in the bioassay medium to provide the complementary Bliβ peptide. The positive control BLic5ΔA1ΔA2+pUC*licA1* was always included in all the tested plates.

The analysis of the plates revealed the presence of several inhibition halos (Figure 24). The comparison of such areas with that of the positive control was used to recognize clones with no activity (or very reduced activity) and clones with apparently increased activity. Still, among the negative clones it was necessary to confirm the presence of *licA1* gene into the vector. This was performed using colony-PCR as described in Appendix 8. After this, 1625 clones incapable of inhibiting the indicator strain (but containing the *licA1* gene) and 90 clones with possible improved properties were identified. Thus, these strains were selected for further analysis.

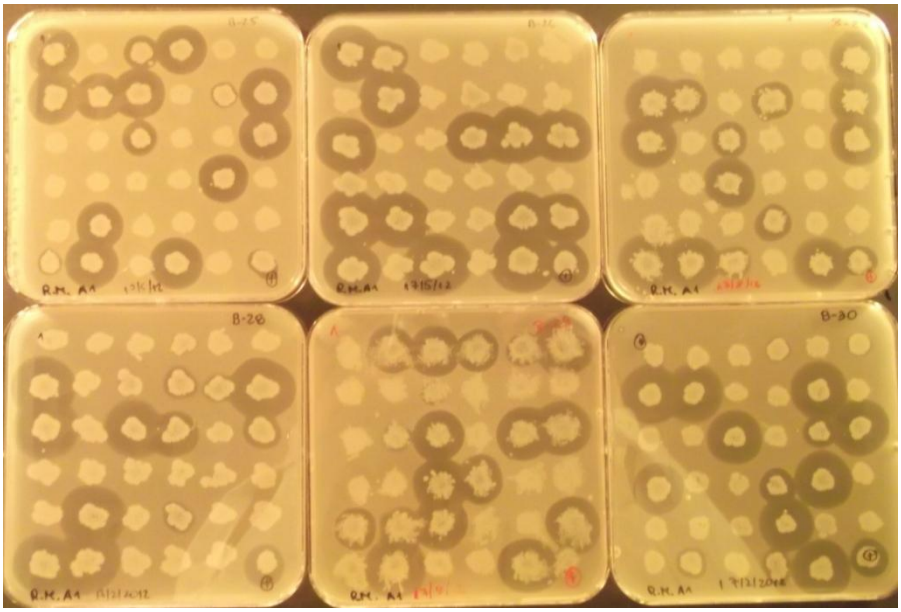

Figure 24 – Example of same bioassay plates; several inhibition halos are visible; comparison of the halo size with these of the positive control allowed to check for increased activity.

4.2.3 Analysis of Bliα mutations

In the present work, the *licA1* nucleotide sequence of only 10 clones was analyzed. Among those, 5 clones possessing no activity (A1.1, A1.10, A1.12, A1.16 and A1.23) and 5 clones with increased activity (A1.13, A5.3, A5.14, A6.30 and A12.15). Before sequencing, a new bioassay was performed to confirm the initial phenotype identified (Figure 25).

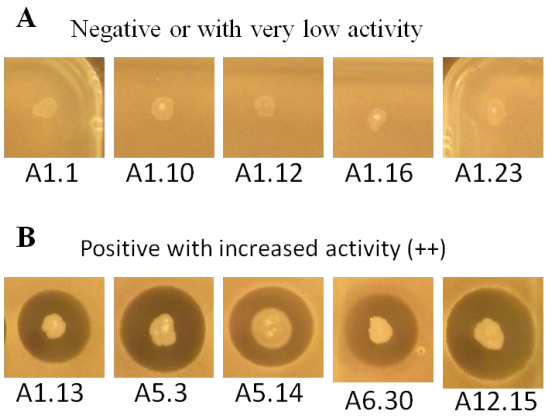

Figure 25 – Bioassay with the clones to be sequenced. (A) negative clones; (B) positive clones with increased activity.

After sequencing, the results were analyzed by comparing both the nucleotide and amino acids sequences with the original *licA1* sequence. With this approach, the

nucleotide mutations that do not influence the amino acid sequence (silent-mutations) could also be identified.

Concerning clones with no activity it is important to mention that the absence of bioactivity can be due to an incorrect production of the peptide. This was observed for clones A1.10 and A1.23. In the first case, a frame shifting mutation was identified. In the second, a stop codon was inserted. Thus, these clones will not be considered for further tests. Regarding the remaining three tested clones, the detected mutations resulted in amino acid substitution as shown in Figure 26.

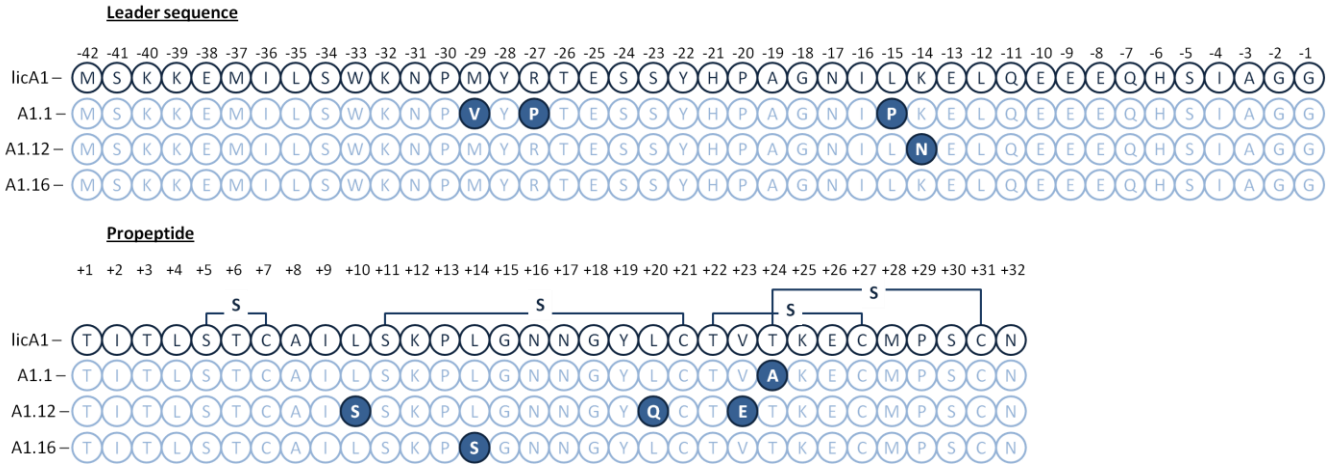

Figure 26 – Comparison between the selected clones and the original *licA1* leader sequence and propeptide.

As shown in Figure 26 each sequence presented at least one mutation in the sequence of the structural gene. However, some of them showed more than one mutation, including mutations in the leader sequence. In such cases, it is difficult to understand if the absence of bioactivity is due to the accumulation of mutations or to a single specific mutation. Thus, if such clones were further studied, other techniques such as site-directed mutagenesis should also be used in order to confirm which mutation(s) is the responsible for the loss of activity.

For clones A1.1 and A1.12, mutations were identified in both the leader sequence and the propeptide. Considering A1.1, the clone possesses a Thr24Ala mutation. In fact, this mutation was already performed in a previous study (Caetano, *et al.*, 2011), which resulted in the complete loss of activity. Such mutation should prevent the formation of a MeLan ring, thus, could contribute to its structural instability, and the phenotype observed should result from the absence of its production as described by Caetano *et al.* (2011).

Regarding the clone A1.12, it is difficult to understand which mutation can cause the observed phenotype. However, it was found that both A1.12 and A1.16 have a Leu→Ser substitution in the propeptide region nearby the ring forming amino acids. Such mutations have been associated with both decreasing and increasing of mersacidin (analogous to Bliα peptide) bioactivity (Appleyard, *et al.*, 2009). In the same study, Leu→Gln substitutions (as observed in clone A1.12) induced the production of low levels of mersacidin. The substitution of Val→Glu was not previously reported.

Further research must be developed to help clarifying the effect of mutations in the leader sequence. However, previous studies with nisin and Pep5 lantibiotics, suggest that mutations into this region may influence the maturation and secretion processes of the final peptide, leading to an abolishment of the activity (Vandermeer, *et al.*, 1994, Neis, *et al.*, 1997).

Regarding, the Bliα producers that seemed to present increased bioactivity, 3 clones did not have any mutation and 1 possessed a silent mutation. Thus, in such cases, the amino acid sequence of the final peptide should not be altered. Such result highlights the unreliability of phenotypic assays to detect improved variants. Only one clone (A1.13) presented a mutation Ser-5Cys. This could be interesting to investigate further once generally the lantibiotic leader sequences do not possess any Cys amino acid. However, analytical data should be obtained for this mutant before assuming that this mutation improves the activity and/or production of Bliα.

4.3 Conclusion

In this chapter a random library of the *licA1* gene was successfully produced and its bioactivity screened. Insertion of the mutations results mainly in non-producing clones.

Random mutagenesis is a useful tool to produce mutants with different levels of bioactivity due to its high frequency of mutation insertion. However, a major drawback of this technique is due to the potential insertion of several mutations simultaneously. This would prevent the complete understanding of which mutation(s) is directly related with a phenotype change, without the application of other complementary analyses such as site-directed mutagenesis. In order to withdraw significant conclusions of the library herein constructed, more clones should be sequenced and the study must be complemented with other analytical methods to ensure more precise outcomes.

4.4 Experimental Procedures

4.4.1 Random Mutagenesis library construction

To perform random mutagenesis it was used GeneMorph II Random Mutagenesis kit (Agilent). Mutazyme II exhibits high misinsertion and misextention frequencies in such a way that mutation rates of 1 to 16 mutations per kb can be achieved using only one set of optimized PCR conditions.

licA1 gene was amplified from pUC*licA1* vector (pUC19a plasmid containing *licA1* gene). A dilution of the pDNA was performed in order to obtain an initial amount of the target gene of 0.1 ng using approximately 1 µL of the template for each reaction. The primers used (Table 9) were mixed together, in order to obtain a final concentration of 250 ng/µL of each primer.

Table 9 – Primers used to amplify *licA1* for random mutagenesis procedure and colony-PCR screening. Represented in bold de recognition site for the restriction enzyme and underlined the start codon.

Primer designation	Primer sequence (5'→3')	Restriction enzyme
licA1_fw_NcoI	TAT**CCATGG**CTATGTCAAAAAAGGAAATG	*Nco*I
licA1_rv_BamHI	TAT**GGATCC**TTAGTTACAGCTTGG CATG	*Bam*HI

The first reaction was performed in a final volume of 50 µL containing 0.5 µL of primermix (250 ng/µL), 1 ng of pDNA, 5 µL of Mutazyme II buffer (10X), 1 µL of dNTP mix (25 mM) and 5 U of Mutazyme II enzyme. The fragment was amplified at 95 ºC for 2 min, followed by 30 cycles of denaturation at 95 ºC for 30 sec, annealing at 50 ºC for 30 sec and extension at 72 ºC for 1 min. The final extension included 10 min at 72 ºC.

After the PCR reaction, 5 µL of the product were analyzed by agarose gel electrophoresis. Afterwards, the mixture of PCR products were purified using NZYtech kit (see Appendix 10) and its concentration was determined using Qubit (Appendix 11). This product was used for a second PCR reaction performed in the same conditions as the first PCR and using 1 ng of DNA.

After purification and DNA quantification, 1000 ng of DNA were digested in a reaction of 60 µL containing 1X of *Bam*HI buffer, 30 U of *Bam*HI and 60 U of *Nco*I restriction enzyme. The mixture was then incubated at 37 ^{o}C for 2 hours. Afterwards, 40 µL of distilled water were added to the reaction and it was purified using the NZYtech

kit. 3 µL of this product were then analyzed by agarose gel electrophoresis. The obtained fragments (150 ng) were ligated to the previously digested pUC19a vector (50 ng) in a reaction containing 1X T4 DNA ligase buffer and 2 µL of T4 DNA ligase enzyme in a final volume of 40 µL. the reaction was allowed to occur at 22 ^{o}C for 1 hour using a thermocycler.

10 µL of the ligation were used to transform 100 µL of chemically competent *E. coli* BLic5ΔA1ΔA2:Apra cells by heat shock (see Appendix 4). After 1 hour at 37 ^{o}C, the culture was centrifuged for 2 min at 6000 xg. The supernatant was then discarded and the pellet was resuspended in 500 µL of LB medium. 100 µl of culture were plated in each LB agar plate containing 12.5 µg/mL of Clo, 50 µg/mL of Apra and 100 µg/mL of Amp. The plates were incubated at 37 ^{o}C overnight.

4.4.2 Library screening by colony-bioassay

Approximately 3000 clones were randomly picked and plated into new LB agar plates supplemented with the appropriate selective markers. In all the plates, the BLic5ΔA1ΔA2+pUC*licA1* strain was also included. Moreover, all plates were identified using a system of number and letters which will allow an easier way of identify each clone: a letter referent to the number of the transformation, a number identifying the number of the plate, followed by a second number indicating the number of the clone. The plates were incubated overnight at 37 ºC.

The bioactivity of each one of the selected clones was performed by colony-bioassay. The plates were prepared as described in section 3.5.3. However, supernatant of the BLic5ΔA1 strain was used instead of that of BLic5ΔA2, in order to provide the complementary Bliβ peptide. The clones were inoculated in the bioassay plates using the replica platting technique. In this case, the isolated colonies were inoculated into the bioassay plates, instead of using extracts. This technique is useful when it is necessary to test several different isolate clones, because it allows transferring several clones at one time. Briefly, a wood block with the same form of the plates was used in conjugation with a sterilized velvet piece. Such apparatus was in contact with the original plate for a few seconds and pressuring for a while and then transferred to the bioassay plate in the same conditions. It is important to take in account the orientation of the plates to make sure that it is possible to identify each clone and relate it with its

own activity in the bioassay. All plates where incubated at 37 ^{o}C overnight and the resulting inhibition areas analyzed and recorded.

Those clones without bioactivity were submitted to colony-PCR reactions using DNA Taq polymerase from Promega (Appendix 8) and M13 universal primers. This allowed to discard eventual clones without the *licA1* gene inserted into the plasmid.

4.4.3 Sequencing of *licA1* mutants

From all the confirmed non-active clones, 40 were initially chosen together with 10 clones with potentially increased bioactivity. Five clones of each group were submitted to nucleotide sequence determination (StabVida, Portugal). The nucleotide sequences were compared with that of the original *licA1* gene, which sequence is available in the GenBank database (accession number AAU25566.1) and using CLC Sequence Viewer 6.

CHAPTER V

SYNOPSIS AND FUTURE PERSPECTIVES

5.1 Involvement of LicR protein in Bliα biosynthesis

LicR protein was initially thought to be implicated in the regulation of lichenicidin biosynthesis, once the deletion of *licR* in *E. coli* lead to a loss of bioactivity, which was found to be related with the absence of functional Bliα peptide (Caetano, *et al.*, 2011). However, during the preliminary tests to check for this hypothesis, an insertion in the *licM1* gene was detected in the *licR* knockout strain, which causes an inactive LicM1 protein that consequently is not able to modify LicA1 to its final conformation. A new knockout mutant was generated and it was possible to observe that Bliα presented antimicrobial activity. The expression levels of *licA1* and *licM1* in the presence and absence of *licR* were evaluated and did not reveal any difference.

Considering that the regulation mechanisms in Gram negative and Gram positive strains are different, it was tried to perform the same study using the original producer strain, *B. licheniformis* I89. However, despite the several protocols attempted it was not possible to obtain a *licR* knockout *B. licheniformis* mutant yet.

5.2 Production of each lichenicidin peptide independently under the control of *E. coli* determinants

The heterologous expression system using *E. coli* has been used already to produce each peptide independently. For that and starting from the fosmid containing the whole gene cluster, a knockout was constructed to one of the structural genes to produce each peptide; in another system, both structural genes were deleted followed by complementation with each one of them separately inserted into a plasmid. In the present study, an attempt was made to insert the genes that are directly involved in the biosynthesis of each peptide (including structural gene, those encoding modification and transport proteins and also a protease in the case of Bliβ) into a cloning vector with *E. coli* determinants.

Concerning the results obtained until now with Bliβ, it seems reasonable to suggest that it is possible to produce each peptide independently using *E. coli* transcriptional and translational determinants. Comparing the expression levels of Bliβ of all the available systems for its production, it appears that the system constructed in this study (BpA2M2TP) shows no advantages, once it was not possible to observe increased activity.

The production of Bliα using the same methodology was not accomplished yet and this needs further investigation in order to understand why, once the host does not to cope well with the inserted vector.

5.3 Generation of Bliα peptides showing bioactivity differences using Random Mutagenesis

Considering not only the academic but also the industrial interest in the production of lichenicidin, the development of studies regarding the enhancement of the expression and/or bioactivity of the lantibiotic is important.

The insertion of mutations within the genes can lead to phenotypic differences including increased or decreased activity or even no activity at all. Several techniques could be used to insert such mutations. In the present work, random mutagenesis was chosen, which allows randomly insertion of one or more mutations within a gene by a PCR using an error-prone polymerase. This technique was found to be useful to produce those mutants showing different levels of bioactivity due to its high frequency of mutation insertion. It is important to notice that some mutations can lead to no changes on bioactivity, once they may change a nucleotide without changing the final amino acid or even changing the amino acid that might not affect greatly the bioactivity of the final peptide.

Random mutagenesis was performed for *licA1* gene, in an attempt to generate Bliα peptides with changed activity. It was clear that the majority of the clones lost the ability to produce the peptide or the produced peptide is not active. This technique needs to be complemented with efficacious screening methods, both phenotypic and, mainly, quantitative.

5.4 Major conclusions of the study

The major findings and conclusions of this thesis are bellow highlighted:

- LicR is not essential for the biosynthesis of Bliα peptide in *E. coli* (Chapter II);
- The strain without *licR* gene presents a higher yield of lichenicidin production, indicating that LicR role in biosynthesis regulation in *E. coli* can be different from that usually described in lantibiotic regulation mechanisms (Chapter II);

– The production of Bliβ peptide in *E. coli* was possible by expressing the genes exclusively involved in its biosynthesis (*licA2M2TP*) under the control of an *E. coli* promoter. However, bioassay and analytic quantification suggested that its levels of production were lower when compared with systems involving the complete *lic* gene cluster (Chapter III);

– Bliα production could not be achieved by cloning the genes exclusively necessary for the biosynthesis (*licA1M1T*) under the control of the T7 promoter (Chapter III);

– A random mutagenesis library containing *licA1* variants was constructed; a first bioactivity screening was performed revealing that the majority of the identified clones lost their bioactivity (Chapter IV).

5.5 Future perspectives

Considering all the results obtained in the present work, it is clear that some aspects would benefit from further investigation, in order to clarify several aspects of lichenicidin biosynthesis in *E. coli* as well as in its natural producer *B. licheniformis* I89.

Firstly, the development of a *licR* knockout *B. licheniformis* I89 mutant will be of most importance to understand if LicR protein can be involved in the regulation of the expression of *licA1*/*licM1* genes. Thus, the protocols must be optimized and novel experiments performed in order to produce such strain. Moreover, the optimization of an efficacious protocol for *B. licheniformis* transformation could open several hypotheses for the study of those Gram positive strains that are hardly transformable.

Other challenge to overcome would be the elucidation of the reasons behind the unsuccessful production of Bliα peptide in *E. coli* when the *licA1M1T* genes were expressed under the control of the T7 promoter (Bliβ peptide was achieved using the same host and the same vector). Also, the development of an improved expression system of lichenicidin in *E. coli* will be of major interest. This would be especially relevant for studies that involve the incorporation of noncanonical amino acids.

Regarding the mutagenesis of Bliα, the identification of mutant peptides with increased bioactivity and/or production constitutes a major advantage. Also from the scientific point of view, those mutations causing changes in the peptides bioactivity are an interesting case of study, both for increased and decreased activity or even null activity. Nevertheless, only preliminary screening of the library was performed in this study. The identification of the mutations behind the phenotypes identified should be performed. Moreover, the structure and production levels of those peptides with interesting properties should be further investigated with analytical techniques such as mass spectrometry. However, these analyses should involve an increased number of replicas for each strain to be examined.

This study opened perspective for future studies, namely regarding the biosynthesis and bioengineering of lantibiotics.

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APPENDICES

7.1 Appendix 1

Preparation of selective agents

The selective agents used in the 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 the present study. NR stands for non required. * Protect from light with foil paper.

Selective agent	Supplier	Stock solution (mg/ml)	Final concentration (µg/ml)	Solvent	Sterilization
Ampicilin	Sigma	100	100	Water	Filtration
Apramycin	AppliChem	50	50	Water	Filtration
Chloramphenicol	BDH	25	12.5	Ethanol	NR
Kanamycin	Gibco	100	50	Water	Filtration
Tetracyclin*	Sigma	20	10	Ethanol	NR

7.2 Appendix 2

General Strains

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

Table S2: List of the general strains used in this study with the reference to their genotype and supplier, when available. ATCC (American Type Culture Collection); 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^rgalendA Hte
***E. coli* BW25113**	JIC	$lacI^+rrnB_{T14}$ΔlacZ WJ16 hsdR514ΔaraBAD AH33 ΔrhaBAD LD78
***E. coli* DH5α**	MUL	F^-*endA1glnV44* thi-1 *recA1relA1gyrA96deoRnupG Φ80dlac*Z Δ M15 *Δ(lacZYAargF)* U169, *hsdR17*(r_K^- m_K^+), λ –
***E. coli* S17-1**		recA pro hsdR RP4-2-Tc::Mu-Km::Tn7
***E. coli* ET12567**		F-*dam*-13::Tn9 *dcm*-6 *hsd*M *hsd*R *zjj*-202::Tn10 *rec*F143 *gal*K2 *gal*T22 *ara*-14 *lac*Y1*xyl*-5 *leu*B6 *thi*-1 *ton*A31 *rps*L136 *his*G4 *tsx*-78 *mtl*-1 *gln*V44
***B. licheniformis*I89**	INETI	Lichenicidin producer (Mendo, *et al.*, 2004)
***B. licheniformis*MW3**	WWM	*B. licheniformis* DSM13 (ΔhsdR1, ΔhsdR2); Lichenicidin producer
***Micrococcus luteus* ATCC 9341**	MUL	Indicator strain

7.3 Appendix 3

General Vectors

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

Vector	Source	MW (Kb)	Selective marker	Observations
pKSV7	UC-USA	6.9	Amp^R, Clo^R	*E. coli*/*Bacillus* shuttle vector. ColE1 and oripE194^{TS}. oripE194 replicates at 32ºC and segregates at 42ºC.
pET-24a(+)	Novagen	5.3	Kan^R	Possess an N-terminal T7●Tag® sequence plus an optional C-terminal. His●Tag® sequence.
pKD20	JIC	6.1	Amp^R	Low copy plasmid encoding the ʎ Red recombinase (ɣ, β, exo), which promote a greatly enhanced rate of recombination when using linear DNA. Possesses an optimized RBS for efficient translation of ɣ and expresses ɣ, β, and exo from the arabinose-inducible P_{araB} promoter. It is also a temperature-sensitive replicon to allow for its easy elimination.
pIJ733	JIC	4.3	$Apra^R$	The $Apra^R$ disruption cassette was cloned into the EcoRV site of pBluescript SK II (+). The cassette is flanked by FRT sites (FLP recognition targets) which allows FLP-mediated excision of the cassette.
pUC19a	Fermentas	2.7	Amp^R	High copy number *E. coli* plasmid; pMB1 replicon; region of *E. coli lac* operon containing a CAP protein binding site, promoter P_{lac}, *lac* repressor binding site and the 5'-terminal part of the *lacZ* gene encoding the N-terminal fragment of beta-galactosidase which can be induced by IPTG, includes *Nco*I and *Nhe*I resticiton sites.

7.4 Appendix 4

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

7.4.1 Preparation of competent cells by calcium-chloride method

Chemically competent cells were prepared using an adaptation of the procedure described by Sambrook and Russell (Russell & Sambrook, 2001). The strain was inoculated in 10 mL of LB medium supplemented with the appropriated selective marker, overnight at 37 ^{o}C, 180 rpm. 50 mL of fresh LB medium supplemented with the appropriate antibiotic were inoculated with the described pre-culture and the culture was grown at 37 ^{o}C, 180 rpm, to an OD_{600nm} of approximately 0,3. The culture was then centrifuged at 4 ^{o}C for 2 min at 6300 xg and the resulting pellet was washed with 13 mL of ice-cold 0.1 M of $MgCl_2$ and centrifuged again as mentioned. 25 mL of 0.1 M $CaCl_2$ solution were used to wash the pellet and the cells were then incubated on ice during 20 min and centrifuged once more as described. Finally the cells were resuspended in 1 mL of cryopreservation buffer ($CaCl_2$ 0.1M, 15 % (v/v) glycerol) which was divided in 50 µL aliquots and stored at -80 ^{o}C until use.

7.4.2 Transformation

An aliquot of 50 µL of the abovementioned stored cells were thawed on ice and the desired DNA was added (~5-100 ng of plasmid DNA or 5 µL of ligation reaction). The mixture was incubated on ice for 15 min and transferred to 42 ^{o}C 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 ^{o}C, 180 rpm, and the culture was centrifuged at 2300 xg for 1 min to collect cells. The most part of supernatant was discharged and the pellet was resuspended in the remaining supernatant. Finally cells were spread on LB agar plates containing the appropriate antibiotic which were incubated at 37 ^{o}C, overnight.

7.5 Appendix 5

Preparation and transformation of electrocompetent *E. coli* cells

7.5.1 Preparation of electrocompetent cells

The desired strain was grown in 10 mL LB medium supplemented with the appropriate selective marker, at 30 ^{o}C, 160 rpm, and overnight. 100 µL of this pre-culture were used to inoculate 10 mL of fresh LB medium containing 20 mM of $MgSO_4$ and the antibiotic. The culture was grown in the same conditions until it reaches an OD_{600nm} of approximately 0.4. The culture was then centrifuged at 3300 xg for 5 min and 4 ^{o}C. The supernatant was discarded and the pelleted cells were resuspended in 10 mL of ice-cold 10 % glycerol by gently mixing. The suspension was centrifuged as above and the same procedure was repeated. After the final centrifugation, the cells were resuspended in 100 µL of 10 % glycerol and kept at 4 ^{o}C until use, since this procedure was always performed in the same day of transformation. The selective markers used in *E. coli* BW25113/pKD20/pLic5 growth were 100 µg/mL of Apra and 12.5 µg/mL of Clo.

7.5.2 Electroporation

The freshly prepared electrocompetent cells were electroporated using a Bio-Rad MicroPulser Electroporator: 50 µL of cells were mixed with 100 to 200 ng of DNA and maintained on ice. The mixture was transferred to a 0.1 or 0.2 cm ice-cold electroporation cuvette and a single pulse was applied using 2.5 kV (the expected time constant was 4.5-4.9 ms). Immediately it was added 1 mL of ice-cold LB medium to the cells and the suspension was incubated at 30 or 37 ^{o}C (depending if replication or segregation of pKD20 was desired, respectively) for 1 hour at 180 rpm. The culture was then centrifuged at 2300 xg for 1 min, resuspended in the remaining supernatant and spread in LB agar plates containing the appropriate antibiotics. The plates were incubated at 30 or 37 ^{o}C overnight. The selection of *E. coli* BW25113/pKD20/pLic5 strains possessing the desired gene interruption was performed with 100 µg/mL of Apra and 12.5 µg/mL of Clo at 37 ºC.

7.6 Appendix 6

Extraction of plasmid DNA

7.6.1 Mini-preparations

The routine extraction of plasmid DNA from *E. coli* was performed with QIAprep Spin MiniPrep Kit (QIAGEN) according to manufacturer's instructions. Briefly, the bacterial culture containing the desired plasmid was grown in LB medium with the appropriate selective marker, at 37 ^{o}C, 180 rpm, overnight. 5 mL of the culture were centrifuged at 6000 xg for 2 min and the supernatant was discharged. The remaining pellet was completely resuspended in 250 µL of Buffer P1 (with RNase A added). 250 µL of Buffer P2 were then added and the suspension was mixed thoroughly by inverting the tube 4-6 times (without vortexing). At that point, if LyseBlue was been added to Buffer P1, the suspension will turn blue. Neutralization was performed by the addition of 350 µL of Buffer N3 and by immediately mixing thoroughly by inverting the tube 4-6 times (until the solution becomes cloudy). The lysate was centrifuged for 5 min at top speed in a table-top microcentrifuge and the supernatant was transferred to a QIAprep spin column. After a centrifugation for 1 min at top-speed the flow-through was discharged, the column was placed back into the same collection tube and washed by the adding of 0.5 mL of Buffer PB and centrifuging for 1 min. The flow-though was discharged and the column was washed again with 0.75 mL Buffer PE (with ethanol added) followed by centrifugation as described. The flow-through was discarded and the column, placed into the same collection tube, was centrifuge for an additional 1 min to remove residual ethanol. Finally the column was transferred to a sterile 1.5 mL microcentrifuge tube and the elution og plasmid DNA was performed by the addition of 40 µL of sterile distilled water to the center of the column, incubation at room temperature for 1 min and centrifugation at top-speed for 1 min.

7.6.2 Maxi-preparations

When a higher concentration of plasmid DNA was required, the extraction was performed from an initial culture of 100 mL grown overnight at 37 ^{o}C, 180 rpm in LB medium supplemented with the appropriate selective marker.

The bacterial culture was centrifuged at 5000 xg for 6 min and the pelleted cells were resuspended in 6 mL of Solution I containing lyzozyme and incubated at room temperature for 5 min. 16 mL of freshly prepared solution II was added to the suspension in order to lysate cells. Then 12 mL of the alkaline Solution III were added and the solution was gently mixed for 3 min. The mixture was incubated on ice for 10 min and then centrifuged at top speed for 15 min at 4 ^{o}C to in order to separate the plasmid DNA from the cells debris and chromosomal DNA.

The supernatant containing the plasmid DNA was recovered avoiding the white precipitate of residual cell debris. 0.6 volume of isopropanol was added to the supernatant and incubated at room temperature for 15 min to precipitate the plasmid DNA. The DNA was recovered by centrifugation at 9600 xg for 15 min and the pellet was washed once with 5 mL of 70 % ethanol. The ethanol was removed by centrifugation, as described above, and completely evaporated. The pellet was resuspended in 1 mL of TE.

The sample was then treated in order to remove RNA by the addition of DNase-free RNase A (Roche) to a final concentration of 2 mg/mL and incubated at 37 ^{o}C for 1 hour. The extraction was performed with Phenol/CIA (Invitrogen): 1 volume of Phenol/CIA was added to remove proteins and the mixture was centrifuged at top speed for 5 min. The upper organic phase was transferred to a new tube and 1/10 volume of sodium acetate and 0.6 volume of isopropanol was added to precipitate nucleic acids. The mixture was incubated at room temperature for 30 min and centrifuged at top speed for 15 min at 4 ^{o}C. The pellet was washed with 500 µL of 70 % of ethanol and a last centrifugation at top speed for 15 min was performed. The ethanol was removed and the tube was air-dried to evaporate the residual ethanol. Plasmid DNA was 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 6 .5.

TE: 10 mM Tris-HCl and 1 mM EDTA, pH 8.0.

7.7 Appendix 7

Extraction of fosmid from *E. coli*

To extract fosmid DNA, columns cannot be used because of DNA large size. However, the protocol can be performed using the reagents from the QIAprep Spin MiniPrep Kit (QIAGEN). Next, two protocols are described to extract fosmid DNA: one using the reagents from the kit and the other one by traditional alkaline lysis. This last one can also be used to extract plasmids when it is required a large amount of recovered product.

7.7.1 Protocol 1: using the reagents from the kit to perform alkaline lysis

The bacterial strain was grown overnight in LB medium with the appropriate antibiotic and 10 mL of the bacterial culture was centrifuged for 1 min at top speed in a to-table centrifuge. The supernatant was discarded and the cell pellet was resuspended in 250 µL of buffer P1 at 4 ^{o}C. The cell lysis was performed by the addition of 250 µL of lysis buffer (P2) and mixing, followed by the addition of 350 µL of P3 buffer. The mixture was centrifuged for 5 min at top speed and the supernatant was transferred to a clean 1.5 mL microcentrifuge tube. 1 volume of phenol:CIA was added and mixed well. Another centrifugation was performed in the same conditions as mentioned before. The aqueous upper phase was collected to a new microcentrifuge tube. 1/10 volume of 0.3 M of NaAc (pH 5.2) and 0.6 volume of isopropanol were then added to the recovered supernatant and the mix was incubated at room temperature for 15 min followed by a centrifugation at 4 ^{o}C, top speed for 15 min. The white pellet formed was washed with 1 mL of 70 % (v/v) ethanol and centrifuged 5 min at top speed. After removal of ethanol, the pellet was air-dried to remove residual ethanol. Finally the pellet was resuspended in 30 µL of sterile distilled water.

7.7.2 Protocol 2: using the traditional alkaline lysis

The first part of the procedure is similar to the one abovementioned. However, the cell pellet was resuspended in 250 µL of Solution I (instead of using the kit´s reagents) containing 100 µg/mL of RNase A added just before use, followed by the addition of 250 µL of Solution II freshly prepared and 350 µL of Solution III. This mixture was centrifuged at top speed for 5 min and the supernatant was transferred into a clean 1.5 mL microcentrifuge tube. 1/10 volumes of Solution III and 0.6 volume of isopropanol were added to the recovered supernatant. The procedure follows as referred above when using the reagents from the kit.

Solution I: 50 mM glucose, 25 mM Tris-HCl (pH= 8) and 10 mM EDTA (pH= 8)

Solution II: 200 mM NaOH, 1% (w/v) SDS. Prepare a stock solution of NaOH (10 M) and a stock solution of SDS (10 %) and prepare the final solution just before use.

Solution III: 3 M potassium acetate, pH 5.5

7.8 Appendix 8

PCR using Promega Taq DNA polymerase

To set up parallel reactions and to minimize the possibility of pipetting errors, it was prepared a PCR master mix by mixing water, buffer, dNTPs, primers and Promega Taq DNA polymerase**.** So all solutions were gently vortex and briefly centrifuged after thawing. A 1.5 mL tube was placed on ice and the following components were added for each 25 µL reaction (Table 10):

Table 10 – PCR reaction using Taq DNA polymerase from Promega.

Component of the reaction	Volume
Forward primer (10 mM)	0.75 µL
Reverse primer (10 mM)	0.75 µL
DNA Template*	~ 1 µL
5x Taq DNA Buffer	5 µL
dNTP Mix, 10 mM each	0.5 µL
Taq DNA polymerase (5 U/ µL)	0.125 µL
Sterile, distilled water	until 25 µL

*To perform colony-PCR, instead of the DNA solution as template, one isolated colony is picked to the mixture. The required final volume is performed with distilled water.

The mixture was then gently vortex, briefly centrifuged and divided into PCR tubes and a single colony was picked and added into the solution. The reactions were then placed in the thermocycler and the following thermal cycling conditions were used (Table 11):

Table 11 – Thermal cycling conditions to perform the PCR with Taq DNA polymerase from Promega.

	Temperature (^{o}C)	Time	Number of cycles
Initial denaturation	95	1-3 min	1
Denaturation	95	45 s	
Annealing	Tm-5*	45 s	30
Extension	72	1 min/kb	
Final extension	72	5-15 min	1

*Annealing temperature based on the average of the primers melting temperatures, which was decreased by 5 degrees.

The reaction product was stored at -20 ^{o}C until further use or immediately run in an electrophoresis gel.

7.9 Appendix 9

Agarose gels handling

7.9.1 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 added before pouring the melted agarose in the running tray. In all gels a DNA marker was included, either 0.5 µg of the DNA Ladder Mix (Fermentas). Electrophoresis was generally performed at 150 V for the desired time and the DNA was analyzed 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 ^{o}C.

7.9.2 Purification of DNA from agarose gels

The purification of DNA from agarose gels was performed using the QIAquick Gel Extraction Kit Protocol (Quiagen), according to the manufacturer's instructions. Briefly: the desired DNA fragment was excised from the agarose gel with a clean scalpel and placed in a 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 ^{o}C for 10 min (or at room temperature 1 hour) until the slice was completely dissolved. 1 volume of isopropanol was added and well mixed; the sample was then applied to a QIAquick spin column placed is a 2 mL collection tube and centrifuged at top speed for 1 min. the flow-through was discarded and the column was placed back to the collection tube. The DNA was washed with 750 µL of Buffer PE and the column centrifuged as referred. The flow-though was discharged and the column was centrifuged for an additional minute to ensure the complete removal of residual ethanol. The column, containing the DNA, was placed in a clean 1.5 mL microcentrifuge tube and the DNA was eluted in 30 to 50 µL of sterile distilled water concerning the subsequent application. The elution is performed after 2 min of incubation at room temperature by centrifugation for 2 min at top speed. The sample was stored at -20 ^{o}C until further use.

7.10 Appendix 10

Purification and concentration of PCR products and restriction digestions

Purification and concentration of PCR products and DNA digestions were performed both using the (1) JETquick Purification Kit (Genomed) and (2) NZYGelpure (NZYtech), according to the manufacturer's instructions.

(1) JETquick Purification Kit (Genomed)

The sample was prepared by the addition of 400 µL of solution H1 to 100 µL of PCR assay (this volume can be achieved by the addition of the necessary volume of sterile distilled water). The sample solution was loaded to a JETquick spin column placed into a 2 mL receiver tube and centrifuged at >12 000 xg for 1 min. The flowthrough was discarded and the column was washed with 500 µL of the reconstituted (with ethanol) solution H2. Another centrifugation was performed using the previously described conditions. The flowthrough was discarded and the JETquick column back into the same receiver tube and the tube was centrifuge once again at maximum speed for 1 min to remove the residual ethanol. Finally the column was placed into a clean 1.5 mL microcentrifuge tube and the DNA was eluted by the addition of 30 to 50 µL of sterile distilled water directly onto the center of the silica matrix of the JETquick spin column and centrifugation at >12 000 xg for 2 min.

(2) NZYGelpure (NZYtech)

The volume of the reaction mixture was transferred to a 1.5 mL microcentrifuge tube and five volumes of Binding Buffer were added and mixed well. The mixture was applied to an NZYTech spin column, incubated at room temperature for 2 min and centrifuged for 1 min at top speed. The flow-through was discarded and 600 µL of Wash Buffer were added to the spin column. After 2 min of room temperature incubation, the column was centrifuge for 1 min and the flow-through was discarded. An additional 1 min centrifugation was performed to remove residual ethanol. The NZYTech spin column was then placed into a clean 1.5 mL microcentrifuge tube and 30 to 50 µL of sterile distilled water were added to the centre of the column. The DNA-containing column was incubated at room temperature for 2 min and then centrifuged for 1 min to elute the DNA. The sample was stored at -20 ^{o}C until further use.

7.11 Appendix 11

Determination of RNA/DNA Concentration using Quant-iTTM assays (Invitrogen)

The Quant-iT™ working solution was made by diluting the Quant-iT™ reagent 1:200 in Quant-iT™ buffer (DNA or RNA reagent and buffer according to the sample to be measured). 199 µL of the working solution were loaded into the assay tubes and 1 µL of sample was added (the final volume must be 200 µL). The mixture was mixed by vortexing 2-3 s and incubated for 2 min at room temperature. The tube was then inserted into the Qubit™ fluorometer and the concentration was calculated following the instructions on the screen.