Chemistry & Biology
Article

Heterologous Expression, Biosynthesis, and Mutagenesis of Type II Lantibiotics from *Bacillus licheniformis* in *Escherichia coli*

Tânia Caetano,[1,2] Joanna M. Krawczyk,[3] Eva Mösker,[3] Roderich D. Süssmuth,[3,*] and Sónia Mendo[1,*]
[1]Department of Biology and CESAM, University of Aveiro, 3810 Aveiro, Portugal
[2]Medinfar–Pharmaceutical Products SA, Amadora, 2700 Venda Nova, Portugal
[3]Institut für Chemie, Technische Universität Berlin, 10623 Berlin, Germany
*Correspondence: suessmuth@chem.tu-berlin.de (R.D.S.), smendo@ua.pt (S.M.)
DOI 10.1016/j.chembiol.2010.11.010

SUMMARY

Lichenicidin is a class II two-component lantibiotic produced by *Bacillus licheniformis*. It is composed of the two peptides Bliα and Bliβ, which act synergistically against various Gram-positive bacteria. The lichenicidin gene cluster was successfully expressed in *Escherichia coli*, thus constituting the first report to our knowledge of a full reconstitution of a lantibiotic biosynthetic pathway in vivo by a Gram-negative host. This system was further exploited to characterize and assign the function of proteins encoded in the biosynthetic gene cluster in the maturation of lichenicidin peptides. Moreover, a *trans* complementation system was developed for expression of Bliα and Bliβ variants in vivo. This contribution will spur future studies in the heterologous expression and engineering of lantibiotics.

INTRODUCTION

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

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

Lantibiotics can be classified based on their maturation pathway and antimicrobial activity (Pag and Sahl, 2002; Willey and van der Donk, 2007). The release of *B. licheniformis* DSM13 (isogenic to ATCC 14760) genome (Rey et al., 2004; Veith et al., 2004) in 2004 allowed the identification of a putative lantibiotic gene cluster, which was recently associated with the lichenicidin production (Begley et al., 2009; Dischinger et al., 2009). According to a previously suggested classification (Willey and van der Donk, 2007), lichenicidin is a two-component lantibiotic, a subgroup of antibacterial class II lantibiotics. In two-component lantibiotics, two different structural genes (*lanA1* and *lanA2*) are expressed and subsequently modified by two different LanM proteins (LanM1 and LanM2). After posttranslational modification both peptides are exported and their leader sequences removed by a single, multifunctional protein possessing an N-terminal protease domain designated LanT. Some of these compounds might undergo an additional N-terminal proteolytic step presumably by the action of an extracellular protease.

Generally, α- and β-peptides of two-component lantibiotics act synergistically to exert their full antibacterial activity. Presently, this group includes eight lantibiotics including haloduracin and lacticin 3147 (Figure 1) (Lawton et al., 2007; McClerren et al., 2006; Ryan et al., 1996).

The lichenicidin complex is active against methicillin-resistant *Staphylococcus aureus* (MRSA) and *Listeria monocytogenes* (Begley et al., 2009; Dischinger et al., 2009). It is believed that two-component lantibiotics mechanism of action involves the interaction of both peptides through the binding to lipid II and formation of pores in the bacterial membrane (Oman and van der Donk, 2009; Schneider and Sahl, 2010).

Apart from their antibacterial activity, other interesting bioactivities have recently been described for lantibiotics, e.g., type III lantibiotics possess antipain activity in a mouse model of neuropathic pain (Meindl et al., 2010). Consequently, during the last years, the evaluation of lantibiotic structure-activity relationships has increased, since the information retrieved can be further used in the rational design of new molecules with improved activity (Cortes et al., 2009). Such studies, using site-directed mutagenesis, have already been conducted in vivo for lacticin 3147 peptides (Cotter et al., 2006) and in vitro for haloduracin (Cooper et al., 2008). This approach was also useful

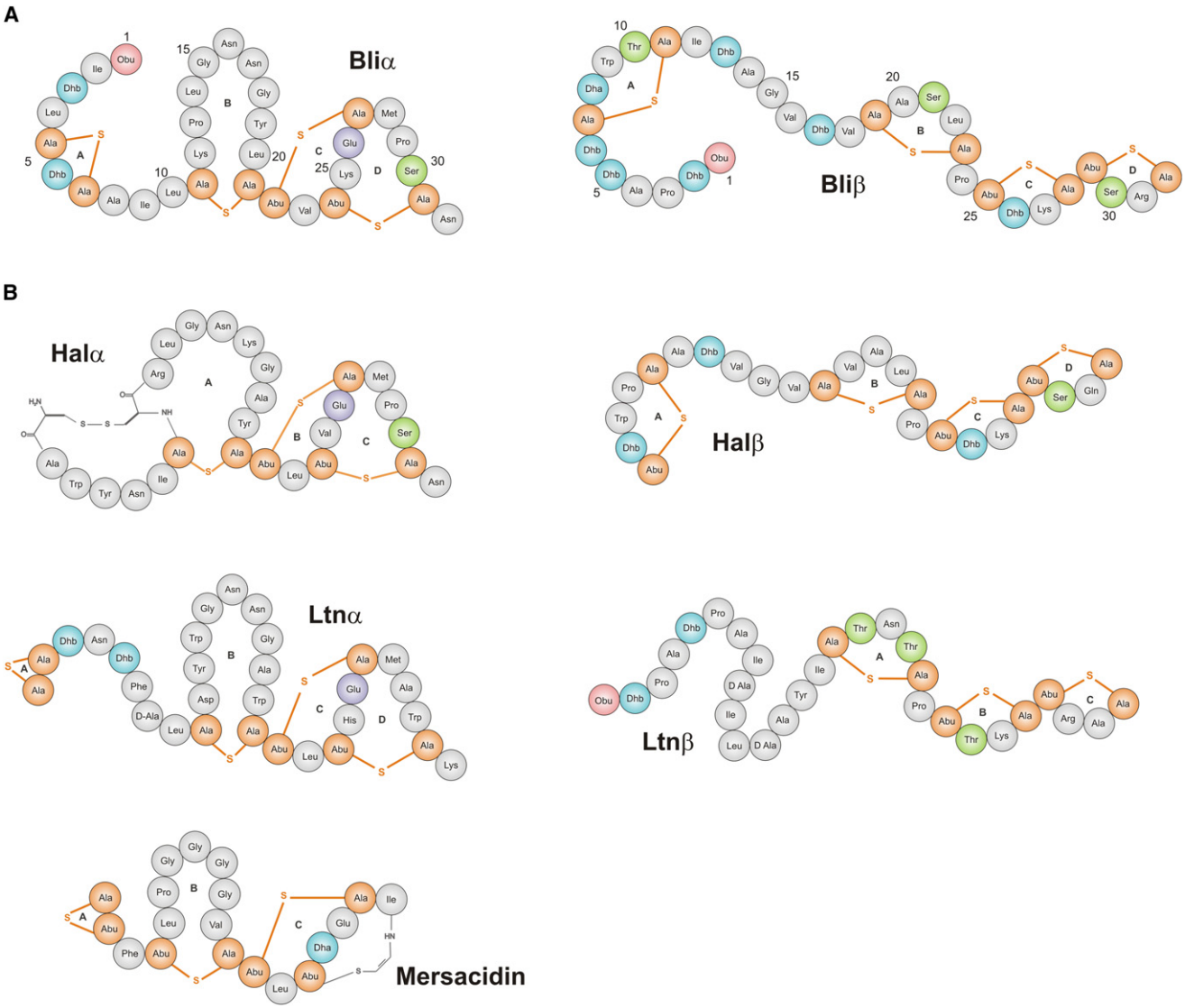

Figure 1. Structure of Class II Lantibiotics

Proposed structures of Bliα and Bliβ peptides of the lichenicidin complex (A) and of the related lantibiotics haloduracin, lacticin 3147 and mersacidin (B). Nondehydrated residues are represented in green and the highly conserved Glu residue in purple (Ala-S-Ala = lanthionine, Abu-S-Ala = methyllanthionine and OBu = 2-oxobutyryl). See also Figures S2–S4.

for the revision of the proposed structure of the haloduracin β-peptide (Cooper et al., 2008). Moreover, a saturation mutagenesis library of mersacidin was recently constructed yielding the production of 82 new compounds in which some of them demonstrated improved activity against Gram-positive pathogens (Appleyard et al., 2009).

Herein, we report the first heterologous expression of a type-II two-component lantibiotic gene cluster in *E. coli* at the example of lichenicidin from *B. licheniformis* I89 (Mendo et al., 2000). Apart from immunity and regulation associated genes the functions of predicted open reading frames in the cluster were assigned. Finally, in *E. coli* a *trans* complementation system was established and new lichenicidin variants were generated, which allowed the assignment of essential residues for expression and antibiotic activity as well as the characterization of structural features previously proposed (Begley et al., 2009). This is the first report to our knowledge on lantibiotic production in Gram-negatives, opening new perspectives for the biotechnological studies of these compounds.

RESULTS

Synthesis of the Two-Component Lantibiotics Bliα and Bliβ by *Bacillus licheniformis* I89

B. licheniformis I89 was previously described as the producer of an antibacterial peptide with a molecular mass of 3249.7 Da (calculated M = 3248.58 Da) (Mendo et al., 2004). In the present study, another antibacterial peptide with the molecular mass of 3019.6 Da (calculated M = 3018.38 Da) was also identified in the extracts of this strain. Both peptides were detected by

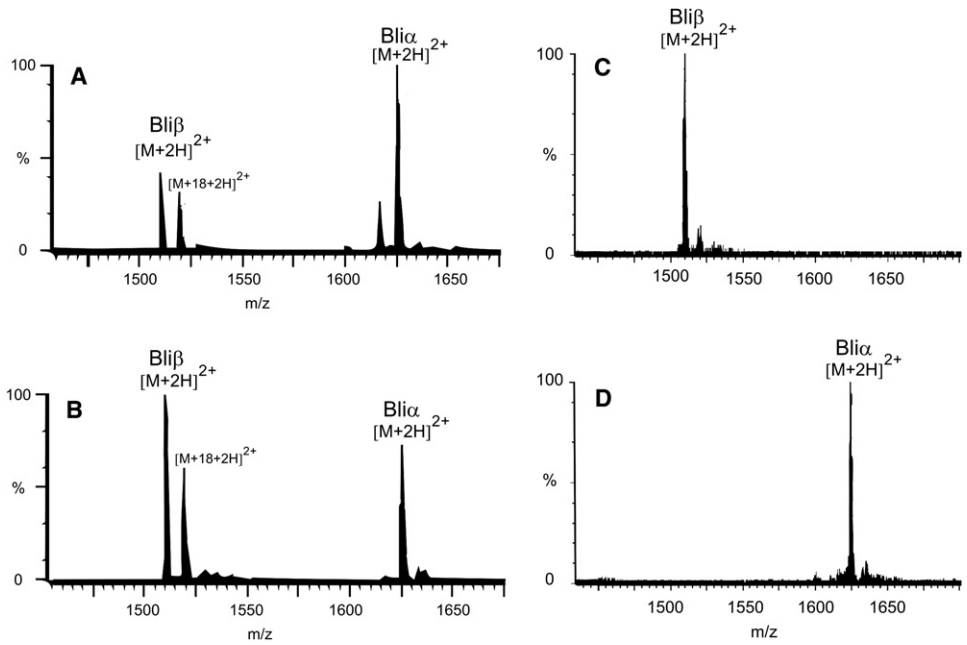

Figure 2. ESI-TOF Mass Spectra of Lichenicidin Peptides

ESI-TOF mass spectra of Bliα (M = 3249.54 Da) and Bliβ (M = 3019.38 Da) peptides detected from *B. licheniformis* I89 supernatant (A) and cell wall washes (B) extracts. *licA1* and *licA2* gene inactivation mutants in Lic5Δ*A1* and Lic5Δ*A2* resulted in the production of only Bliβ (C) and Bliα (D), respectively.

LC-ESI-MS analysis of cell wall washes and supernatant extracts from cultures grown at 37°C (Figure 2) and 50°C. In the *B. licheniformis* ATCC 14760 genome a putative two-component lantibiotic gene cluster was identified (Rey et al., 2004). The products of the structural genes *licA1* and *licA2* matched with the abovementioned molecular masses assuming 7 and 12 dehydration reactions of both propeptides. Recently, Begley et al. (2009) and Dischinger et al. (2009) reported that lichenicidin peptides (Bliα and Bliβ) production is encoded in this cluster. We propose that the antibacterial peptides isolated from *B. licheniformis* I89 are also lichenicidin and therefore the lichenicidin nomenclature will always be adopted.

The analysis of *B. licheniformis* I89 extracts also revealed the presence of at least two additional molecules with molecular masses corresponding to variously dehydrated Bliβ peptides (Figure 2).

Heterologous Expression of Lichenicidin in *Escherichia coli*

B. licheniformis I89 exhibited low transformation efficiencies, probably due to class I restriction modification systems previously identified in *B. licheniformis* DSM13 (Rey et al., 2004; Veith et al., 2004). This is a severe impediment for the molecular biology procedures necessary for gene function analysis and also for rapid access to mutant strains. Thus, heterologous expression of lichenicidin was attempted in the Gram-negative host *Escherichia coli*. To access the complete gene cluster in a vector, a fosmid library of *B. licheniformis* I89 genomic DNA was constructed in *E. coli* EPI300 and screened with a DIG-labeled DNA probe encoding the lichenicidin structural genes *licA1* and *licA2*. Five positive clones (Lic5, Lic7, Lic8, Lic10, and Lic45) were identified, containing the *lic* gene cluster (approximately 14 Kb) (Figure 3A). All the clones inhibited the growth of *M. luteus* and therefore one clone (Lic5) containing the fosmid pLic5 (Figure 3B) was selected as lichenicidin heterologous expression system. Lichenicidin production by Lic5 was evaluated along 36 hr in liquid cultures. LC-ESI-MS analysis of both cell isopropanol washes and supernatant extracts after 24 hr of growth revealed the presence of peptides with molecular masses corresponding to Bliα and Bliβ. The exciting observation of successful heterologous expression of the *lic* cluster in a Gram-negative organism required closer consideration and experimental proof. Hence, detection of Bliα and Bliβ observed in *E. coli* supernatants could either be explained by bacterial lysis of the producing strain or by active export through the outer membrane. Since growth behavior of *E. coli* carrying the *lic* gene cluster was not conspicuous the latter hypothesis was investigated, analyzing the influence of TolC, an outer membrane protein commonly involved in the export of toxins on lichenicidin production. Therefore, *E. coli* BW25113 possessing a deletion of the *tolC* gene was transformed with the fosmid pLic5 carrying the *lic* cluster. The resulting *E. coli* BW25113Δ*tolC:kan*-pLic5 strain did not show antibacterial activity against the indicator strain *M. luteus* (see Figure S6 available online) and the molecular masses of Bliα and Bliβ were not detected by ESI-LC-MS analysis of supernatant extracts. *Trans* complementation of this strain with *tolC* resulted in restoration of antibacterial activity (Figure S6). This was further corroborated by detection of characteristic masses of Bliα and Bliβ by means of HPLC-ESI-MS from supernatant extracts (data not shown).

Mutagenesis of the Lichenicidin Biosynthetic Cluster

The *lic* biosynthetic gene cluster (Figure 3A) is composed of 14 open reading frames (ORFs). Expression of these genes in

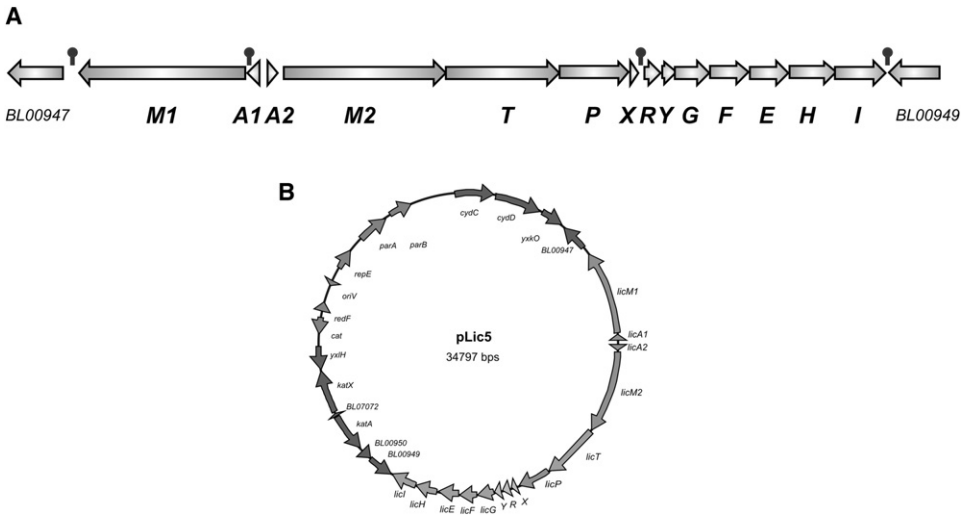

Figure 3. Lichenicidin Gene Cluster and pLic5 Map

(A) Organization of the lichenicidin biosynthetic gene cluster, according to the genome annotation for *Bacillus licheniformis* ATCC 14760. Black circles correspond to deduced Rho-dependent terminators.

(B) Representation of the pLic5 fosmid map, containing the lichenicidin biosynthetic gene cluster.

See also Table S1.

E. coli Lic5 further permitted the construction of gene-inactivated mutants using the λ RED recombinase system (Datsenko and Wanner, 2000; Gust et al., 2003). To that end, the fosmid pLic5 was introduced into BW25113 cells containing the RED recombinase expression plasmid pKD20 (Datsenko and Wanner, 2000). The transformed strain was used as a platform for the inactivation of all ORFs of the *lic* cluster, except those presumably associated with self-immunity. In total, nine fosmids were constructed, each of them containing the deletion of one gene in BW25113 cells and transformed into EPI300 cells (Table S1). Overall, the expression of eight ORFs was found to be critical for lichenicidin production (Figure 4A). Only the Lic5*ΔX* knockout mutant retained visible antibacterial activity against the indicator strain *M. luteus* (Figure 4A).

According to previous studies on two-component lantibiotics, it was expected that the single production of either Bliα or Bliβ would result in significantly reduced antibacterial activity. Consequently, the loss of activity by mutational inactivation of *lic* genes could result from the absence of production of both or only one of the lichenicidin peptides. If the second assumption was correct, antibacterial activity should be restored if the complementing peptide could be externally supplied by cross-feeding. Indeed, this was observed when the two strains missing the respective intact structural genes (Lic5*ΔA1* and Lic5*ΔA2*) were bioassayed as neighboring colonies (Figure 4B). Moreover, no *M. luteus* inhibition was observed when Lic5*ΔA1* mutant was inoculated next to Lic5*ΔA1*, or Lic5*ΔA2* next to Lic5*ΔA2*. LC-ESI-MS analysis of both clones revealed that Lic5*ΔA1* synthesized exclusively the Bliβ peptide and Lic5*ΔA2* synthesized exclusively the Bliα peptide (Figures 2C and 2D). These results showed that inhibition of *M. luteus* was a consequence of the synergistic activity of both peptides. Therefore, Lic5*ΔA1* and Lic5*ΔA2* were used in cross-feeding agar-diffusion assays with the other knockout mutants. Full restoration of antibacterial activity was observed between the following combinations of knockout mutant pairs: Lic5*ΔA1* with Lic5*ΔM2*, Lic5*ΔP* and Lic5*ΔR*; Lic5*ΔA2* with Lic5*ΔM1* and Lic5*ΔY* (Figure 4C). These results were predictive of peptide production in each knockout mutant and were unambiguously confirmed by LC-ESI-MS analysis (Table S1).

LC-ESI-MS analysis of the mutants obtained from inactivation of dehydratase-cyclases Lic5*ΔM1* and Lic5*ΔM2*, showed the same results as those obtained with the mutants of the structural genes Lic5*ΔA1* and Lic5*ΔA2*, respectively. Molecular masses corresponding to propeptides or prepropeptides were not detected.

Sequence homology studies identified LicT from the *B. licheniformis* I89 *lic* cluster as a member of the ABC transporter family with an integrated protease domain, presumably responsible for removal of the leader sequences of Bliα and Bliβ during substrate translocation. In *E. coli*, the inactivation of *licT* (Lic5*ΔT*) resulted in the loss of visible antibacterial activity, which was not restored by interaction with Lic5*ΔA1* or Lic5*ΔA2*. Accordingly, molecular masses of Bliα and Bliβ peptides were not identified in the Lic5*ΔT* supernatant extracts by LC-ESI-MS. However, when isopropanol washes of Lic5*ΔT* cells were analyzed by LC-ESI-MS a peptide mass corresponding to fully processed Bliβ was identified, albeit in extremely low concentration. It is likely that isopropanol treatment of Lic5*ΔT* cells released Bliβ propetide to interact with the protease LicP (active in this mutant), thus yielding correctly processed Bliβ peptide without the leader sequence.

The *licX* gene encodes a small uncharacterized hypothetical protein with significant homology to other uncharacterized *Bacillus* sp. proteins. The absence of conserved core motifs in this protein makes it difficult to predict its function. When *licX* was inactivated, the antibacterial activity of Lic5*ΔX* was not affected and both peptides Bliα and Bliβ were identified in

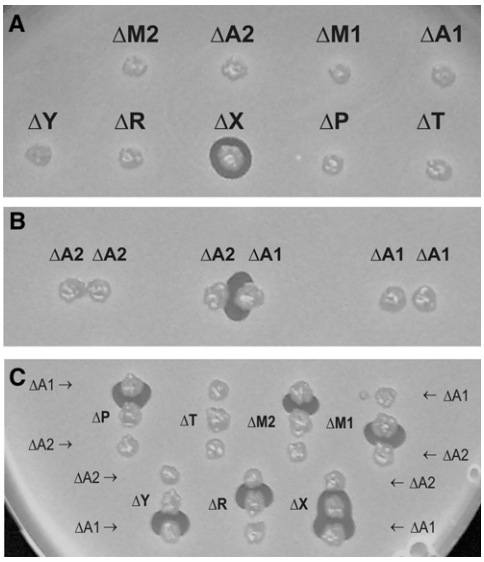

Figure 4. Bioactivity of Knockout Mutants

Agar diffusion assay to assess lichenicidin production by the knockout mutants of the *lic* gene cluster using *M. luteus* as the indicator strain. Lic5 gene inactivation mutants are represented by *ΔA1*, *ΔA2*, *ΔM1*, *ΔM2*, *ΔT*, *ΔP*, *ΔX*, *ΔR* and *ΔY*, according with the inactivated gene. (A) Antibacterial activity exhibited by all knockout mutants produced in this study. (B) Synergetic activity of peptides Bliα and Bliβ, produced by Lic5*ΔA2* and Lic5*ΔA1*, respectively. (C) Restored lichenicidin activity of the knockout mutants upon interaction with Bliβ and Bliα peptides produced by Lic5*ΔA1* and Lic5*ΔA2*, respectively. See also Figure S1.

LC-ESI-MS analysis, indicating that LicX does not play an essential role in lichenicidin production.

Downstream of *licX*, two other genes, *licR* and *licY*, were identified. NCBI Conserved Domain Searches of the *licR* sequence revealed a high homology to helix-turn-helix XRE family-like proteins. After its deletion only Bliβ was identified by LC-ESI-MS analysis of Lic5*ΔR* extracts. This result demonstrates that LicR might have an essential regulatory function in the biosynthesis of mature LicA2 peptide. Finally, the Lic5*ΔY* mutant was identified as the only producer of the Bliα peptide. This proved an essential involvement of LicY in the biosynthesis of Bliβ but not in the biosynthesis of Bliα. Three transmembrane helices identified in LicY point to a likely role as a membrane protein.

LicP, A Protease Involved in Bliβ Biosynthesis

Bliα and Bliβ prepropeptides both have a common proteolytic site for LicT (C-terminal of the Gly-Gly-motif). The molecular mass of the processed Bliβ peptide affords additional proteolysis of an N-terminal hexapeptide of the Bliβ' peptide (corresponding to H_2N-NDVNPE-Bliβ). Such second proteolysis step has been described for other two-component lantibiotics, e.g., Halβ, Plwβ, $CylL_L$, and $CylL_S$ (Booth et al., 1996; Holo et al., 2001; McClerren et al., 2006). In the *lic* gene cluster, *licP* encodes a putative uncharacterized serine protease homologous to CylA (38% identity) from *Enterococcus faecalis* ($CylL_L$ and $CylL_S$ producer) and to BH1491 uncharacterized protease (30% identity) from *B. halodurans* C-125 (Halβ producer). Therefore, it was considered that LicP could be the protease postoperative to LicT responsible for the removal of the hexapeptide NDVNPE from Bliβ'. In fact, when *licP* was deleted only the production of Bliβ was negatively affected. The *E. coli* Lic5*ΔP* mutant lost antibacterial activity, which could be restored upon cross feeding with a mutant delivering correctly processed Bliβ peptide e.g., mutant Lic5*ΔA1* (Figure 4C). Hence, in Lic5*ΔP* isopropanol washes of cells and supernatant extracts only the molecular mass of the Bliα was identified. Molecular masses corresponding to the N-terminally untrimmed Bliβ peptide (calculated M = 3686.65 Da) or those of its derivatives could not be identified. However, when *E. coli* Lic5*ΔP* cell and supernatant extracts where bioassayed next to filter-sterilized supernatants of an *E. coli* strain solely expressing LicP (BL21 *licP*$^+$), the reestablishment of antibacterial activity was observed (Figure S1). This indicated that fully active Bliβ was produced in situ and could act synergistically with the Bliα peptide.

A Complementation System for Expression of Lichenicidin Peptide Derivatives in *E. coli*

The construction of a system enabling generation of lichenicidin variants was developed, due to its usefulness for both structure-activity relationship (SAR) analysis and structure elucidation purposes. To that end, *E. coli* BL21Gold producing exclusively one of the prepeptides were obtained by transformation of these cells with pLic5*ΔA1* or pLic5*ΔA2* fosmids, generating BLic5*ΔA1* and BLic5*ΔA2* strains, respectively. These strains were complemented with the respective deleted genes (*licA1* and *licA2*) cloned into the expression vector pET-24a(+). The resulting transformants BLic5*ΔA1*$^+$ (BLic5*ΔA1*+p*licA1*) and BLic5*ΔA2*$^+$ (BLic5*ΔA2*+p*licA2*) were able to inhibit the indicator strain and the molecular masses of both Bliα and Bliβ by LC-ESI-MS were successfully identified. Therefore, this system was further exploited by PCR site-directed mutagenesis of p*licA1* and p*licA2* in order to obtain *E. coli* strains producing Bliα and Bliβ variants. During this procedure, only one of the lichenicidin peptides was mutated at each time, facilitating an exact assignment of the impact of the introduced alteration also in production and/or bioactivity. The generation of 32 lichenicidin variants was attempted, which molecular masses are summarized in the supplementary information (Table S2).

In this study a systematic Ala scan was performed for all Cys, Ser, and Thr residues of Bliα and Bliβ. Especially C-terminal substitutions of residues BliαThr24, BliβThr25, BliβCys28, and BliβThr29A did not yield the corresponding mutant peptide (HPLC-ESI-MS control) and consequently no bioactivity was observed (Figure 5). All other lichenicidin variants were synthesized although to a different extent. Some of the peptides variants were produced in low amounts and therefore were not used in bioactivity assays (Figure 5). Peptides which were produced in amounts comparable to the wild-type (HPLC-ESI-MS control) were correlated to bioactivity in agar diffusion tests. Overall, the changes introduced at the N terminus of Bliα and Bliβ seem to be less prejudicial for lichenicidin bioactivity and/or for peptide production than those at the C terminus (Figure 5).

Homology studies of mersacidin-like two-component lantibiotics revealed the presence of a highly conserved glutamate (Glu17) (Figure 1). Its substitution in mersacidin, Halα and Ltcα completely abolished antibacterial activity (Appleyard et al.,

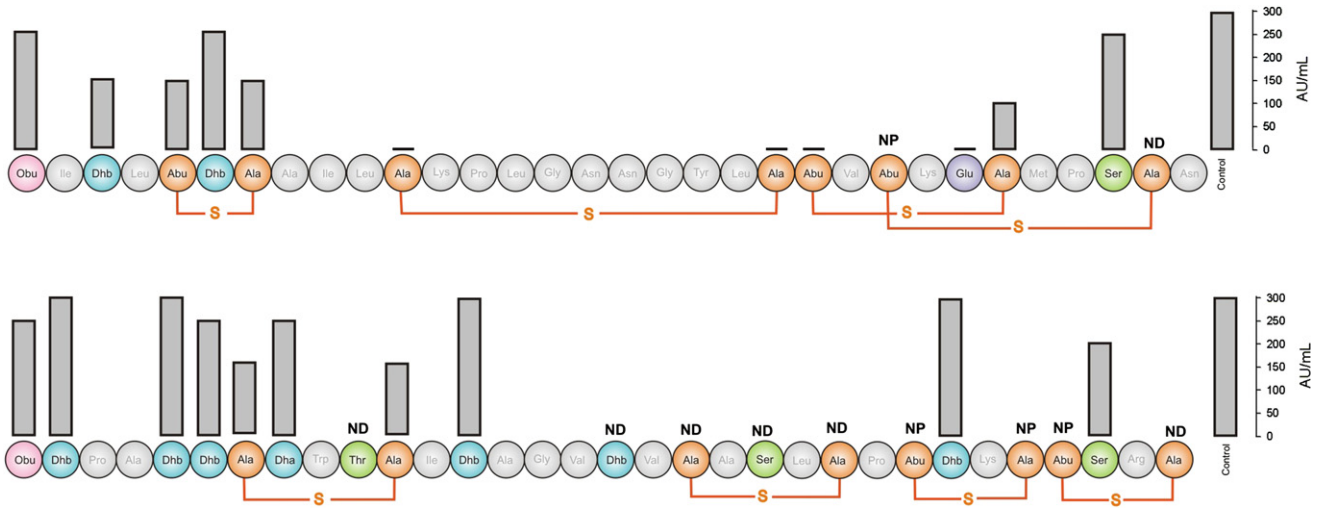

Figure 5. Lichenicidin Alanine-Scan Mutagenesis

Supernatant extracts activity (AU.ml^{-1}) of Bliα (A) and Bliβ (B) alanine-scan mutants against *M. luteus*. Control strains represent the mutants BLic*ΔA1* and BLic*ΔA2*, complemented with p*licA1* (A) and p*licA2* (B). The primary amino acid sequence and thioether rings of Bliα and Bliβ are represented underneath the bioactivity results, accordingly with the results obtained in this study (OBu = 2-oxobutyryl). Nonproducer (NP) strains or low-producer (ND) strains of mutated peptides were not integrated in the bioassay. See also Table S2.

2009; Cooper et al., 2008; Cotter et al., 2006). For mersacidin it was proved that Glu17 is essential for binding to the lipid II target (Brotz et al., 1998; Hsu et al., 2003; Szekat et al., 2003). In the case of lichenicidin Bliα, the substitution of homologous Glu26 by Ala yielded the expected peptide (M = 3191.54 Da), however antibacterial activity of BliαE26A was completely abolished.

Localization of the N-Terminal Lanthionine Bridges of Bliα and Bliβ

The determined exact molecular masses of Bliα and Bliβ peptides (M = 3249.54 Da and M = 3019.6 Da, respectively) differ from the calculated masses by +1 Da, suggesting that a 2-oxobutyryl residue could be present at the N terminus of both peptides. This hypothesis was also supported by mutant peptides of Bliα and Bliβ possessing an N-terminal Thr→Ala replacement thus retaining a conventional N terminus (BliαT1A: M = 3236.52 Da and BliβT1A: M = 3006.36 Da) and bioactivity. Hence, establishment of N-terminal A-rings of Bliα and Bliβ between Dhb1 and Cys7 (Bliα) and Dhb1 and Cys11 (Bliβ) seemed less likely, contrary to data previously proposed by Begley et al. (2009). MS/MS analysis of the Bliα peptide from *B. licheniformis* I89 (Figure 6) and *E. coli* BLic5 (Figure S3) supernatants showed the presence of the N-terminal ions a_3 and b_3, excluding the possibility of A-ring formation between Cys7 and Dhb1 or Dhb3. Instead, either Dha5 or Dhb6 were candidates for A-ring formation with Cys7. This question was further investigated by MS/MS experiments of Bliα peptides containing an Ala5 (BliαS5A mutant) or Thr6 (BliαT6A mutant). The identification of the y_{26} ion for BliαS5A but not for BliαT6A supports a Bliα structure with an A-ring between Dha5 and Cys7 (Figure 1A; Figure S4). The MS/MS spectra of the Bliβ peptide from *B. licheniformis* I89 (Figure 6) and *E. coli* BLic5 (Figure S3) supernatant permitted the identification of a series of fragment ions (a_2, b_2, a_3, b_3, b_5, b_6, y_{27}, and y_{26}), suggesting that only Dha7 or Dhb8 were likely to be involved in A-ring formation with Cys11. The analysis of the bioactivity of the peptides possessing an Ala residue instead of Dhb2, Dhb5, Dhb6, Dha7, and Dha8 suggests that Dha7 should be the residue reacting with Cys11 of Bliβ peptide (Figures 1A and 5).

Localization of Didehydro Amino Acids in the Structures of Bliα and Bliβ Peptides

The found molecular masses of Bliα and Bliβ peptides are consistent with the posttranslational dehydration of seven and twelve Ser/Thr residues, respectively. Therefore, one and three Ser/Thr should escape the action of the dehydratase-cyclases LicM1 and LicM2, respectively. These residues were proposed to be Ser30 of Bliα and Ser21, Thr26 and Ser30 of Bliβ (Begley et al., 2009). To confirm this assumption experimentally, these four residues were mutated to Ala. Analysis of the BliαS30A mutant revealed the presence of a 7-fold dehydrated peptide (M = 3233.58 Da). Therefore, Ser30 from Bliα escapes dehydratation. The same analysis for Bliβ mutants BliβS21A and BliβS30A revealed products with molecular masses corresponding to the expected twelve dehydratations (M = 3003.40 Da). Therefore, LicM2 does not modify Ser21 and Ser30. However, the peptide produced by BliβT26A mutant (M = 3007.40 Da) was consistent with the occurrence of only eleven, instead of the proposed twelve dehydratations (calculated M = 2989.37 Da) (Begley et al., 2009). The substitution of Thr10→Ala resulted in the synthesis of a 12-fold dehydrated peptide (M = 2989.72 Da) (Figure S2 and Table S2). Thus, Thr10 is the candidate amino acid escaping LicM2 dehydration and this finding was also included in the Bliβ structure (Figure 1A).

DISCUSSION

In the light of recent developments in the field of lantibiotic biosynthesis, the role of lantibiotics for use as antimicrobials is promissory. This is supported by observations that when

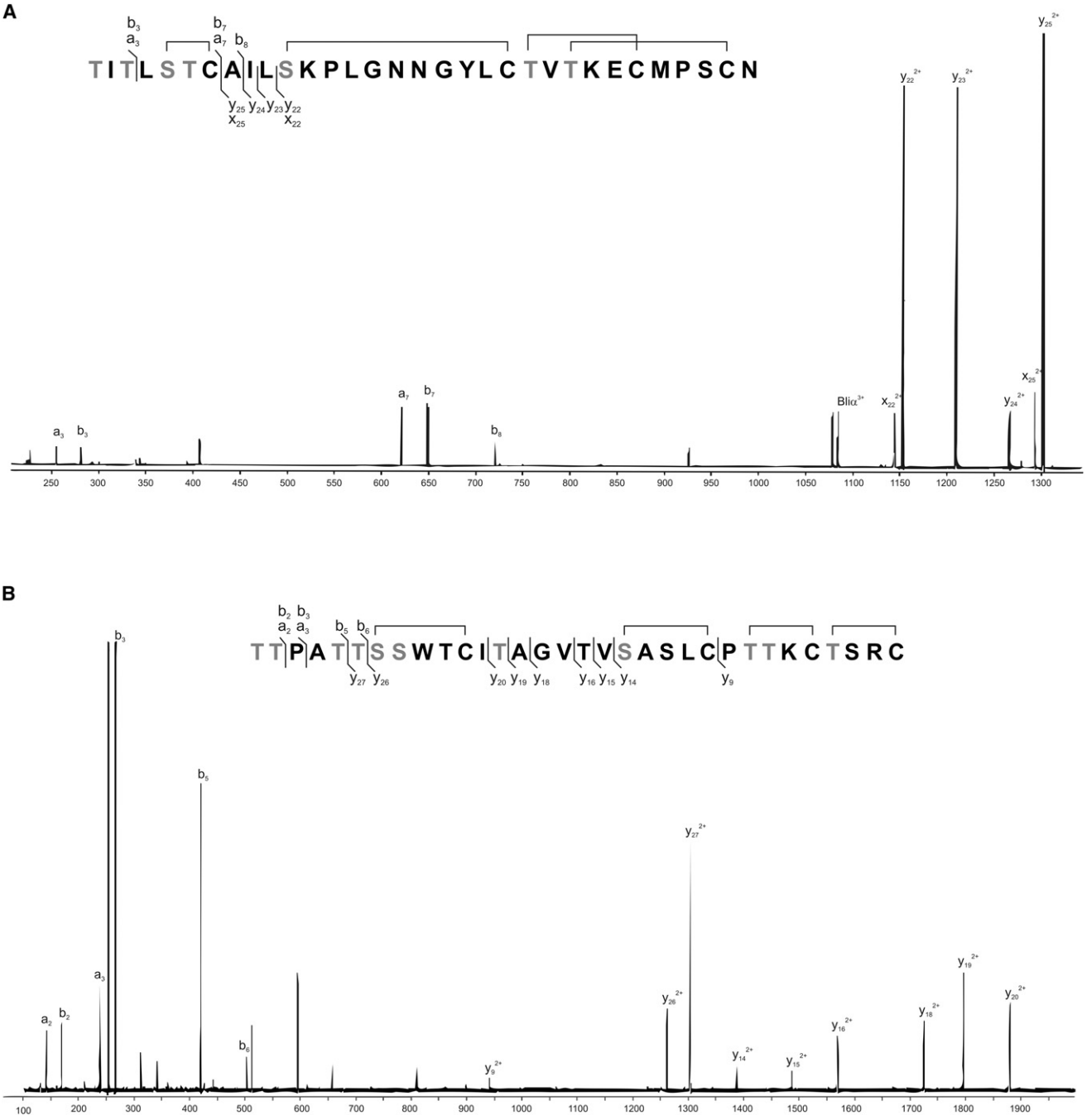

Figure 6. Bliα and Bliβ ESI-MS/MS Spectra

ESI-MS/MS spectra of Bliα (A) and Bliβ (B) purified from the *B. licheniformis* I89 supernatant and assigned fragmentation pattern of lichenicidin peptides. See also Figure S4.

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

B. licheniformis I89 was not amenable to transformation and therefore heterologous expression of lichenicidin was attempted

in *E. coli*. This Gram-negative organism is a preferred heterologous expression host with respect to genetic manipulation, handling cost and time considerations. The presence of the fosmid p*Lic5* harboring the complete lichenicidin gene cluster in *E. coli* (Lic5) resulted in a strain able to inhibit *M. luteus* growth. Characteristic molecular masses of fully modified Bliα and Bliβ peptides were detected by LC-ESI-MS in *E. coli* Lic5 cell washes (isopropanol) and supernatant extracts. In the past, the heterologous production of lantibiotics in *E. coli* hosts was unknown. Recently, first steps into this direction where undertaken by the co-expression of prepeptide and the corresponding modifying enzyme in *E. coli*, which achieved establishment of the fully dehydrated lantibiotic BovHJ50 (Lin et al., 2010). However, its biologically active form was only obtained after in vitro incubation with a specific protease (BovT150) to remove the leader sequence. In contrast, herein we obtained a full reconstitution of the in vivo synthesis of a lantibiotic in a Gram-negative host. Moreover, heterologous expression of the *lic* cluster in a *tolC*-deficient *E. coli* strain revealed the absence of Bliα and Bliβ peptides in supernatant extracts. This supported the assumption that TolC and/or TolC-related proteins are likely candidates for the transport of Bliα and Bliβ through the outer membrane, which was further corroborated by restoration of lichenicidin activity upon *tolC trans*-complementation.

The lichenicidin gene cluster consists of 14 ORFs and in the past, only the expression of *licM1* and *licM2* genes was associated with lichenicidin production (Dischinger et al., 2009). Therefore, λ RED recombinase technology was employed to inactivate all *lic* genes in the p*Lic5* fosmid, except those putatively related with immunity. Our results showed that *licA1* and *licA2* encode the lichenicidin prepeptides, which are posttranslationally modified by bifunctional dehhydratases-cyclases LicM1 and LicM2, respectively. In the absence of LicT *(E. coli* Lic5*ΔT)*, it was not possible to detect the fully processed Bliα and Bliβ peptides in supernatant extracts, indicating that LicT protein plays an essential role in the maturation of both peptides. LicT protein is a member of the ABC transporter family with an integrated protease domain, apparently responsible for the removal of the leader sequences of Bliα and Bliβ during their transport. *E. coli* proteases and toxins are often exported directly from the cytoplasm to the supernatant by a *sec*-independent type I secretion system (T1S). T1S affords the presence of an ABC transporter, a membrane fusion protein (MFP) and an outer membrane protein (OMP; e.g., TolC) (Kostakioti et al., 2005). Considering this system, it could be hypothesized that LicT located in the inner membrane is able to closely interact with MFP and TolC (or TolC-related protein) of *E. coli*, forming a T1S-complex responsible for leader peptide removal and export of Bliα and Bliβ. It is also conceivable, that LicT removes the leader peptides and transports Bliα and Bliβ to the periplasmatic space followed by TolC-mediated export without any interaction between LicT and TolC. Alternatively, Bliα and Bliβ could be exported by inner and outer membrane transporters provided by the *E. coli* host. Then LicT would only contribute a proteolytic function required for leader peptide removal. Nevertheless, these hypotheses have to be further evaluated experimentally in order to establish a comprehensive model for lantibiotics secretion in Gram-negative hosts.

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

The LicX sequence is similar to other uncharacterized hypothetical proteins of the *Bacillus* genus and no conserved motifs could be identified, making it difficult to predict its function. Yet, we could establish that LicX is not involved in the Bliα and Bliβ biosynthesis or its function could be complemented when using *E. coli* as the heterologous host. LicR is a predicted DNA-binding protein with high similarity (60%) to other transcriptional regulators from *Bacillus* genus. The *licR* gene inactivation proved the exclusive involvement of LicR in Bliα production, most probably through the induction of *licA1* transcription. Likewise, disruption of a similar gene, *mrsR1* present in the mersacidin biosynthetic cluster strongly decreased its prepeptide transcription rate resulting in the absence of MrsA production (Guder et al., 2002; Schmitz et al., 2006). Since Bliα and MrsA peptides are highly homologous (Figure 1) and are produced by *Bacillus* species, it seems possible that their regulation mechanisms have evolved from a common ancestor. The LicY protein has been found essential for production of the Bliβ peptide, but not for production of Bliα. LicY does not display significant similarities to other proteins available in databases, making it impossible to assign a functional role to this protein. Like CylR1 of the cytolysines (Coburn and Gilmore, 2003) the LicY secondary structure is predicted to contain three transmembrane helices and a function as a membrane-bound protein seems likely. CylR1 and CylR2 have been described as a two-component regulatory system, which represses the expression of cytolysin genes through a quorum-sensing mechanism (Haas et al., 2002). Nevertheless, it is difficult to apply this model to the regulation of Bliβ expression, since it was not possible to identify a gene encoding DNA-binding protein within the *lic* biosynthetic cluster, which could be associated with Bliβ production. In this study, we assigned for the first time to our knowledge the association of two putative distinct regulatory elements to the production of one specific single peptide, Bliα or Bliβ, of the lichenicidin two-component system. We hypothesize, that analogous mechanisms of regulation are involved in the biosynthesis of other two-component lantibiotics, e.g., of haloduracin. In fact, the proteins BH0460 and BH0459 encoded downstream of *hal* biosynthetic gene cluster show high homology to LicR (60%) and LicY (61%), respectively. Further studies examining gene cluster transcription and protein interaction will shed light on the assignment of the exact role of LicR and LicY on lichenicidin production.

The successful heterologous expression of the *lic* gene cluster in *E. coli* facilitated the establishment of a system for the

expression of lichenicidin variants. The peptides resulting from Ala substitutions and subsequent MS/MS analysis (of the wild-type or mutated peptides) allowed the revision of some structural features of both Bliα and Bliβ previously proposed by (Begley et al., 2009). Compared with the structurally related two-component lantibiotics haloduracin and lacticin, the high number of serines and threonines in lichenicidin propeptide sequences, particularly at the N termini, made a clear assignment of dehydrated residues difficult. The Ala-scan performed for all Ser- and Thr-containing positions confirmed that Ser30 escapes dehydration in Bliα and likewise, Thr10, Ser21, and Ser30 remain unmodified in the Bliβ peptide. Herein, it was established that LicM2 dehydrates Thr26 of Bliβ, similarly to the homologous residue in Halβ (Dhb18), but in contrast to Lctβ (Thr23). The exact molecular masses obtained for Bliα and Bliβ exceed those of previously determined peptides by +1 Da, indicating that after proteolysis, a spontaneous deamination of Dhb1 to Obu1 occurs in both Bliα and Bliβ, as described for Pep5 and Lctβ peptides (Ryan et al., 1999). This is clearly supported by mass spectrometry of peptides BliαT1A and BliβT1A suggesting that Thr1 of both peptides is not involved in A-ring formation. The fragmentation pattern of lichenicidin wild-type peptides (Figure 6) rather indicates that thioether rings are formed between Ser5 or Thr6 with Cys7 (Bliα) and Ser7 or Ser8 with Cys11 (Bliβ). MS/MS spectra of BliαS5A and BliαT6A, identified Bliα Ser5 as the amino acid involved in the Bliα A-ring formation. For Bliβ the bioactivity of BliβS7A suggests that Ser7 is the residue for Lan formation with Cys11. Recently, the structure of lichenicidin peptides Lchα and Lchβ isolated from *B. licheniformis* VK21 were elucidated by NMR (Shenkarev et al., 2010). In general, the proposed structures are in agreement with the present work except for the A-ring of Bliα peptide. The MS/MS spectra obtained for Bliα of both *B. licheniformis* I89, *B. licheniformis* DSM13 and *E. coli* BLic5 exclude the occurrence of a thioether ring between Thr3 and Cys7 due to the presence of an a_3-ion. Therefore, Bliα and Lchα may represent natural variants of lanthionine formation. Yet there is no experimental evidence that minor differences in dehydratase-cyclase could generate a structural variability, however, cluster alignment of LicM1 from *B. licheniformis* I89 (HQ290360) and *B. licheniformis* VK21 showed the presence of a 6 aa difference between both modifying enzymes (Figure S5).

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

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

SIGNIFICANCE

This is the first report, to our knowledge, of a lantibiotic biosynthesis pathway totally reconstituted in vivo in the heterologous host *E. coli*, using a vector containing the whole lichenicidin biosynthetic gene cluster. This system was used to generate knockout mutants for all the genes of the lichenicidin biosynthetic gene cluster, except for ORFs involved in lichenicidin immunity. The following conclusions could be drawn: (1) LicP is a protease most probably responsible for the Bliβ trimming, which activity seems to be independent of the LicT action; (2) LicX is not essential for lichenicidin production; and (3) LicR and LicY are involved exclusively in Bliα and Bliβ biosynthesis. Furthermore, an in vivo *trans* complementation system was developed which allowed production of lichenicidin peptides with a variety of amino acid substitutions. This study opens up the possibility for more complex yet time efficient structure-activity relationship studies on lichenicidin and may initiate new research efforts in lantibiotic biosynthesis investigation and bioengineering using Gram-negative hosts.

EXPERIMENTAL PROCEDURES

The bacterial strains, plasmids, general growth conditions, general reagents used in molecular biology procedures and antibacterial assays are fully described in Supplemental Experimental Procedures.

Lichenicidin Heterologous Expression in *E. coli*

The description of the fosmid library construction is present in the supplemental information. *E. coli* clones positive for *lic* structural genes were screened for lichenicidin production by colony bioassay. The production stability without selective pressure was assessed through sequential subcultures of *E. coli* clones tested against the indicator strain. Based on these assays and sequencing, clone Lic5 was selected and its lichenicidin production evaluation was performed in medium M, as follows: 1 ml samples were taken every 2 hr, along 36 hr, from 50 ml cultures and centrifuged for 5 min at 16,100 × g. The cell-free supernatant was stored at −20°C while cells were resuspended in 100 μl of 70% isopropanol containing 0.1% of formic acid. After 2 hr at 4°C, cells were centrifuged for 5 min at 16,100 × g and the supernatant stored at −20°C. Forty microliters of each of the samples was used for bioassays. Samples exhibiting the major inhibition zone were selected for LC-ESI-MS analysis. Negative controls consisting of *E. coli* EPI300 possessing the empty pCC2FOS vector were also included in this analysis.

Lichenicidin Gene Cluster Knockout Mutants

In-frame gene deletions of lichenicidin biosynthetic cluster were performed using the λ-Red-mediated recombination system adapted from (Gust et al., 2003). In brief, the fosmid pLic5 was used to transform electrocompetent BW25113 cells containing the pKD20 plasmid (BW25113/pKD20/pLic5). Disruption cassettes for each gene were amplified using as DNA template the 1383 bp fragment resulting from the digestion of pIJ733 ($Apra^R$) with EcoRI/HindIII. All the designed primers possess two *Bmt*I restriction sites at each side of the $Apra^R$ cassette (Table S4). At least 100 ng of PCR product were used to transform electrocompetent BW25113/pKD20/pLic5 cells. Positive clones were screened by PCR using the respective check primers (Table S4). Fosmids containing the desired Apra cassette inserted were extracted from 50 ml overnight cultures using the alkaline lysis procedure (Sambrook and Russell, 2001) and digested with *Bmt*I (New England Bioloabs) for 3 hr. Restriction enzyme was removed by phenol/CIA extraction, followed by DNA precipitation with 1/10 volumes of KAc and 0.6 volumes of isopropanol. *Bmt*I-digested fosmid was religated in a 50 μl reaction during 15 min at 20°C

using T4 DNA ligase. Religation (5 μl) was used to transform chemically competent EPI300 cells. Positive clones were screened by colony-PCR and tested for the Cm^R and $Apra^S$ phenotype.

LicP Proteolysis Analysis

General procedures involved in the analysis of LicP proteolysis are described in Supplemental Experimental Procedures.

trans Complementation System

The *licA1* and *licA2* coding regions were amplified with the primers described in the primers described in Table S4. PCR products and pET24-a(+) vector were digested with BamHI and *Xho*I. After restriction enzymes removal, *licA1* and *licA2* digests were ligated to pET24-a(+) to form the p*licA1* and p*licA2* plasmids. Five microliters of the ligation reaction was used to transform chemically competent DH5α cells. Positive clones were screened with T7prom and T7terminator primers and corresponding plasmids extracted with the GeneJET Plasmid Miniprep Kit (Fermentas). Fifty nanograms of p*licA1* and p*licA2* was used to transform chemically competent BLic5Δ*A1* and BLic5Δ*A2* cells, respectively. Clo^R and Kan^R clones were confirmed to be positive by PCR. The restoration of lichenicidin antibiotic activity was assessed by colony bioassay against *M. luteus*. Lichenicidin peptides production was confirmed by LC-ESI-MS analysis of cell and supernatant extracts.

Production of Lichenicidin Variants

Alanine scanning of Bliα and Bliβ peptides was performed by site-directed mutagenesis of *licA1* and *licA2* genes, using a modification of the two-step reactions method described by (Wang and Malcolm, 1999), using p*licA1* and p*licA2* as templates. Primers used to insert the desired mutation were designed in the web-based program PrimerX. 5 cycles of amplification were used in the first PCR and 20 cycles in the second. The parental methylated DNA was digested with 10 U of *Dpn*I, followed by transformation in DH5α. Three different Kan^R clones were selected for plasmid extraction. The insertion of the desired mutation in each plasmid was confirmed by sequencing reaction using T7prom and T7term primers. Finally, mutated p*licA1* and p*licA2* plasmids were transformed in chemically competent BLic5Δ*A1* and BLic5Δ*A2* cells, respectively. Production of Bliα and Bliβ variants was confirmed by LC-ESI-MS analysis of total extracts (cell and supernatant) and the same samples were used for antibacterial activity assays.

Preparation of Lichenicidin Extracts for Analysis

For lichenicidin production evaluation, 50 ml cultures in medium M were performed at 37°C with aeration during 24 hr. The cells were pellet by centrifugation at 13,000 × g for 5 min, dissolved in 2 ml of 70% isopropanol containing 0.1% formic acid and stored at 4°C during 3 hr. The cell wall washes extract was recovered by centrifugation at 13,000 × g for 5 min and stored at −20°C. 2:10 volumes of 1-butanol was added to the cell-free supernatant , and the mixture was stirred for 1 hr at room temperature and finally centrifuged at 3000 × g for 1 min. Supernatant extracts corresponding to the upper organic phase were collected and stored at −20°C. For alanine-scan mutants, total extracts were analyzed. These were obtained by addition of 1:4 volumes of 1-butanol to 20 ml of bacterial culture and sonicated for 15 min. After 1 hr of stirring, the culture was centrifuged at 4000 × g for 5 min and the organic phase was collected. The solvent was evaporated and pellets diluted in 70% ACN/dH2O.

Mass Spectrometry Analysis

Routine analytics was performed on a qTof 2 hybrid quadrupol time of flight ESI mass spectrometer (Micromass/Waters, Milford, MA) coupled to an Agilent 1100 HPLC system (Agilent, Waldbronn, Germany). HR-ESI-MS experiments were obtained on an Orbitrap XL massspectrometer (Thermo Scientific, Bremen, Germany) coupled to an Agilent 1200 HPLC system (Agilent Technologies, Waldbronn, Germany). Separations were performed using a column Eclipse XDB C-18, 5 μm, 4.6*150 mm (Agilent) with a linear solvent gradient (5% methanol (0.1% HFo) to 100% methanol (0.1% HFo) in 25 min staying 10 min at 100% organic solvent). MS/MS experiments were performed on an Agilent triple quadrupole Massspectrometer 6460 coupled with an Agilent 1290 HPLC, equipped with Agilent thermostatted column compartment SL+, autosampler and binary pump. The injection of pure compounds was accomplished via loop injection. For extracts, an Agilent column Eclipse plus C18 RRHD 1.8 μm, 2.1 × 50 mm was used for separations (gradient from 5% acetonitril to 100% acetonitril in 6 min staying 2 min at 100% acetonitril). Fragment voltage and collision energy was optimized by each sample.

ACCESSION NUMBERS

Coordinates have been deposited in the GenBank database with accession numbers HQ290359 and HQ290360.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and three tables and can be found with this article online at doi:10.1016/j.chembiol.2010.11.010.

ACKNOWLEDGMENTS

T.C. was supported by Fundação para a Ciência e Tecnologia and Medinfar Pharmaceuticals SA grant (SFRH/BDE/15559/2005). The work was supported by Centre for Environmental and Marine Studies (CESAM) of University of Aveiro and the Cluster of Excellence "Unifying concepts in catalysis" granted by the DFG, coordinated by TU Berlin. The authors acknowledge Dr. José C. Duarte for supplying *B. licheniformis* I89 strain, and Professor Dr. Klaus Hantke from University of Tübingen for supplying *E. coli* BW25113 and BW25113Δ*tolC:kan* strains.

Received: July 7, 2010
Revised: October 19, 2010
Accepted: November 4, 2010
Published: January 27, 2011

REFERENCES

Appleyard, A.N., Choi, S., Read, D.M., Lightfoot, A., Boakes, S., Hoffmann, A., Chopra, I., Bierbaum, G., Rudd, B.A.M., Dawson, M.J., et al. (2009). Dissecting structural and functional diversity of the lantibiotic mersacidin. Chem. Biol. *16*, 490–498.

Begley, M., Cotter, P.D., Hill, C., and Ross, R.P. (2009). Identification of a novel two-peptide lantibiotic, lichenicidin, following rational genome mining for LanM proteins. Appl. Environ. Microbiol. *75*, 5451–5460.

Booth, M.C., Bogie, C.P., Sahl, H.-G., Siezen, R.J., Hatter, K.L., and Gilmore, M.S. (1996). Structural analysis and proteolytic activation of *Enterococcus faecalis* cytolysin, a novel lantibiotic. Mol. Microbiol. *21*, 1175–1184.

Brotz, H., Bierbaum, G., Leopold, K., Reynolds, P.E., and Sahl, H.G. (1998). The lantibiotic mersacidin inhibits peptidoglycan synthesis by targeting lipid II. Antimicrob. Agents Chemother. *42*, 154–160.

Chatterjee, C., Paul, M., Xie, L., and van der Donk, W.A. (2005). Biosynthesis and mode of action of lantibiotics. Chem. Rev. *105*, 633–684.

Coburn, P.S., and Gilmore, M.S. (2003). The *Enterococcus faecalis* cytolysin: a novel toxin active against eukaryotic and prokaryotic cells. Cell. Microbiol. *5*, 661–669.

Cooper, L.E., McClerren, A.L., Chary, A., and van der Donk, W.A. (2008). Structure-activity relationship studies of the two-component lantibiotic haloduracin. Chem. Biol. *15*, 1035–1045.

Cortes, J., Appleyard, A.N., and Dawson, M.J. (2009). Whole-cell generation of lantibiotic variants. Methods Enzymol. *458*, 559–574.

Cotter, P.D., Deegan, L.H., Lawton, E.M., Draper, L.A., O'Connor, P.M., Hill, C., and Ross, R.P. (2006). Complete alanine scanning of the two-component lantibiotic lacticin 3147: generating a blueprint for rational drug design. Mol. Microbiol. *62*, 735–747.

Datsenko, K.A., and Wanner, B.L. (2000). One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc. Natl. Acad. Sci. USA *97*, 6640–6645.

Dischinger, J., Josten, M., Szekat, C., Sahl, H.-G., and Bierbaum, G. (2009). Production of the novel two-peptide lantibiotic lichenicidin by *Bacillus licheniformis* DSM 13. PLoS One *4*, e6788.

Guder, A., Schmitter, T., Wiedemann, I., Sahl, H.G., and Bierbaum, G. (2002). Role of the single regulator MrsR1 and the two-component system MrsR2/K2 in the regulation of mersacidin production and immunity. Appl. Environ. Microbiol. *68*, 106–113.

Gust, B., Challis, G.L., Fowler, K., Kieser, T., and Chater, K.F. (2003). PCR-targeted *Streptomyces* gene replacement identifies a protein domain needed for biosynthesis of the sesquiterpene soil odor geosmin. Proc. Natl. Acad. Sci. USA *100*, 1541–1546.

Haas, W., Shepard, B.D., and Gilmore, M.S. (2002). Two-component regulator of *Enterococcus faecalis* cytolysin responds to quorum-sensing autoinduction. Nature *415*, 84–87.

Holo, H., Jeknic, Z., Daeschel, M., Stevanovic, S., and Nes, I.F. (2001). Plantaricin W from *Lactobacillus plantarum* belongs to a new family of two-peptide lantibiotics. Microbiology *147*, 643–651.

Hsu, S.-T.D., Breukink, E., Bierbaum, G., Sahl, H.-G., de Kruijff, B., Kaptein, R., van Nuland, N.A.J., and Bonvin, A.M.J.J. (2003). NMR study of mersacidin and lipid II interaction in dodecylphosphocholine micelles. J. Biol. Chem. *278*, 13110–13117.

Kostakioti, M., Newman, C.L., Thanassi, D.G., and Stathopoulos, C. (2005). Mechanisms of protein export across the bacterial outer membrane. J. Bacteriol. *187*, 4306–4314.

Lawton, E.M., Cotter, P.D., Hill, C., and Ross, R.P. (2007). Identification of a novel two-peptide lantibiotic, haloduracin, produced by the alkaliphile *Bacillus halodurans* C-125. FEMS Microbiol. Lett. *267*, 64–71.

Lin, Y., Teng, K., Huan, L., and Zhong, J. (2010). Dissection of the bridging pattern of bovicin HJ50, a lantibiotic containing a characteristic disulfide bridge. Microbiol. Res. 10.1016/j.micres.2010.05.001.

McClerren, A.L., Cooper, L.E., Quan, C., Thomas, P.M., Kelleher, N.L., and van der Donk, W.A. (2006). Discovery and in vitro biosynthesis of haloduracin, a two-component lantibiotic. Proc. Natl. Acad. Sci. USA *103*, 17243–17248.

Meindl, K., Schmiederer, T., Schneider, K., Reicke, A., Butz, D., Keller, S., Gühring, H., Vértesy, L., Wink, J., Hoffmann, H., et al. (2010). Labyrinthopeptins: a new class of carbacyclic lantibiotics. Angew. Chem. Int. Ed. Engl. *49*, 1151–1154.

Mendo, S.A.L.V., Henriques, I.S., Correia, A.C.M., and Duarte, J.M.C. (2000). Genetic characterization of a new thermotolerant *Bacillus licheniformis* strain. Curr. Microbiol. *40*, 137–139.

Mendo, S., Faustino, N.A., Sarmento, A.C., Amado, F., and Moir, A.J.G. (2004). Purification and characterization of a new peptide antibiotic produced by a thermotolerant *Bacillus licheniformis* strain. Biotechnol. Lett. *26*, 115–119.

Oman, T.J., and van der Donk, W.A. (2009). Insights into the mode of action of the two-peptide lantibiotic haloduracin. ACS Chem. Biol. *4*, 865–874.

Pag, U., and Sahl, H.G. (2002). Multiple activities in lantibiotics—models for the design of novel antibiotics? Curr. Pharm. Des. *8*, 815–833.

Rey, M.W., Ramaiya, P., Nelson, B., Brody-Karpin, S., Zaretsky, E., Tang, M., de Leon, A., Xiang, H., Gusti, V., Clausen, I.G., et al. (2004). Complete genome sequence of the industrial bacterium Bacillus licheniformis and comparisons with closely related Bacillus species. Genome Biol. *5*, r77.

Ryan, M.P., Rea, M.C., Hill, C., and Ross, R.P. (1996). An application in cheddar cheese manufacture for a strain of *Lactococcus lactis* producing a novel broad-spectrum bacteriocin, lacticin 3147. Appl. Environ. Microbiol. *62*, 612–619.

Ryan, M.P., Jack, R.W., Josten, M., Sahl, H.-G., Jung, G., Ross, R.P., and Hill, C. (1999). Extensive post-translational modification, including serine to D-alanine conversion, in the two-component lantibiotic, lacticin 3147. J. Biol. Chem. *274*, 37544–37550.

Sambrook, J., and Russell, D.W. (2001). Molecular cloning: a laboratory manual (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

Schmitz, S., Hoffmann, A., Szekat, C., Rudd, B., and Bierbaum, G. (2006). The lantibiotic mersacidin is an autoinducing peptide. Appl. Environ. Microbiol. *72*, 7270–7277.

Schneider, T., and Sahl, H.G. (2010). Lipid II and other bactoprenol-bound cell wall precursors as drug targets. Curr. Opin. Investig. Drugs *11*, 157–164.

Shenkarev, Z.O., Finkina, E.I., Nurmukhamedova, E.K., Balandin, S.V., Mineev, K.S., Nadezhdin, K.D., Yakimenko, Z.A., Tagaev, A.A., Temirov, Y.V., Arseniev, A.S., et al. (2010). Isolation, structure elucidation, and synergistic antibacterial activity of a novel two-component lantibiotic lichenicidin from *Bacillus licheniformis* VK21. Biochemistry *49*, 6462–6472.

Stein, T. (2005). *Bacillus subtilis* antibiotics: structures, syntheses and specific functions. Mol. Microbiol. *56*, 845–857.

Szekat, C., Jack, R.W., Skutlarek, D., Farber, H., and Bierbaum, G. (2003). Construction of an expression system for site-directed mutagenesis of the lantibiotic mersacidin. Appl. Environ. Microbiol. *69*, 3777–3783.

Veith, B., Herzberg, C., Steckel, S., Feesche, J., Maurer, K.H., Ehrenreich, P., Bassumer, S., Henne, A., Liesegang, H., Merkl, R., et al. (2004). The complete genome sequence of *Bacillus licheniformis* DSM13, an organism with great industrial potential. J. Mol. Microbiol. Biotechnol. *7*, 204–211.

Wang, W., and Malcolm, B. (1999). Two-stage PCR protocol allowing introduction of multiple mutations, deletions and insertions using QuikChange site-directed mutagenesis. Biotechniques *26*, 680–682.

Willey, J.M., and van der Donk, W.A. (2007). Lantibiotics: peptides of diverse structure and function. Annu. Rev. Microbiol. *61*, 477–501.

