Characterization of Amylolysin, a Novel Lantibiotic from Bacillus amyloliquefaciens GA1

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Abstract

Background: Lantibiotics are heat-stable peptides characterized by the presence of thioether amino acid lanthionine and methyllanthionine. They are capable to inhibit the growth of Gram-positive bacteria, including Listeria monocytogenes, Staphylococcus aureus or Bacillus cereus, the causative agents of food-borne diseases or nosocomial infections. Lantibiotic biosynthetic machinery is encoded by gene cluster composed by a structural gene that codes for a pre-lantibiotic peptide and other genes involved in pre-lantibiotic modifications, regulation, export and immunity.

Methodology/Findings: Bacillus amyloliquefaciens GA1 was found to produce an antimicrobial peptide, named amylolysin, active on an array of Gram-positive bacteria, including methicillin resistant S. aureus. Genome characterization led to the identification of a putative lantibiotic gene cluster that comprises a structural gene (amlA) and genes involved in modification (amlM), transport (amlT), regulation (amlKR) and immunity (amlFE). Disruption of amlA led to loss of biological activity, confirming thus that the identified gene cluster is related to amylolysin synthesis. MALDI-TOF and LC-MS analysis on purified amylolysin demonstrated that this latter corresponds to a novel lantibiotic not described to date. The ability of amylolysin to interact in vitro with the lipid II, the carrier of peptidoglycan monomers across the cytoplasmic membrane and the presence of a unique modification gene suggest that the identified peptide belongs to the group B lantibiotic. Amylolysin immunity seems to be driven by only two AmlF and AmlE proteins, which is uncommon within the Bacillus genus.

Conclusion/Significance: Apart from mersacidin produced by Bacillus amyloliquefaciens strains Y2 and HIL Y-85,544728, reports on the synthesis of type B-lantibiotic in this species are scarce. This study reports on a genetic and structural characterization of another representative of the type B lantibiotic in B. amyloliquefaciens.

Introduction

Bacteriocins are antimicrobial peptides ribosomally synthesized capable to inhibit the growth of Gram-positive bacteria, including Listeria monocytogenes, Staphylococcus aureus or Bacillus cereus, the causative agents of food-borne diseases or nosocomial infections [1,2]. The class I bacteriocins, the so-called lantibiotics, are heat stable post-translationally modified peptides that contain multiple thioether amino acids lanthionine (Lan) and methyllanthionine (Melan) [3]. These latter are respectively enzymatically synthesized from a cysteine thiol and the dehydrated dehydroalanine (Dha) or dehydrobutyrine (Dhb) amino acids [4].

Lantibiotics could be subdivided into two main subgroups: type-A lantibiotics that exhibit a linear secondary structure and are positively charged at neutral pH. They are modified by two distinct LanB and LanC enzymes and processed by a LanP protease. Type-B lantibiotics, conversely, exhibit a globular structure and are non-charged or slightly negatively charged at neutral pH. They are modified by a single modification LanM enzyme and processed by a LanT ABC transporter with N-terminal-associated protease activity [5]. Type-B subgroup also includes the so-called two-component lantibiotics consisting of
two synergistically acting peptides that are modified by a single LanM-type enzyme [6]. Recently, a third Type-C subgroup has been reported. It corresponds to peptides, such as SapT and SapB from Streptomyces tendae, that present mainly a morphogenetic function rather than an antimicrobial activity. Beside this, lantibiotic could also be distinguished based on their biological mode of action. Some, such as mersacidin, bind to lipid II and thereby inhibit peptidoglycan cell wall synthesis in sensitive Gram-positive bacteria [7] while others, such as Pep5, form pores in cytoplasmic membrane that lead to cell leakage and finally to cell death [8,9]. A third group of compounds is formed by lantibiotic that possess a dual mode of action, i.e. inhibition of the peptidoglycan biosynthesis and pore forming. Both mode of action could be ensured by a single peptide (such as nisin) or by two distinct peptides in two-component lantibiotic, such as lacticin 3147 [10].

Lantibiotic peptides are gene encoded and their structural genetic determinants are found in biosynthetic gene clusters. They are synthesized as inactive prepeptides, with a N-terminal leader sequence separated from the mature lantibiotic [3]. In type A lantibiotics, many of the leader end with an EP or PR sequence and share a conserved F(N/D)LD motif in their core sequence. On the other hand, leader peptides of type-B lantibiotics end with G(G/A/S) sequence [6]. They are not processed by a LanP protease such as in type-A lantibiotic but by the N-terminal intracellular domain of LanT protein [11]. In addition, all lantibiotic gene clusters encode a set of immunity proteins that protect the producer strain against the biological effect of the synthesized lantibiotic. Depending on the lantibiotic considered, these immunity proteins could be LanI, LanF, LanE or LanG [12]. Regulation of lantibiotics biosynthesis is mediated by regulatory LanR and LanK proteins that constitute a two-component signal transduction system [13].

Despite the majority of the lantibiotics described so far are from lactic bacteria, Bacillus represents an alternative genus to investigate for antimicrobial peptides because it includes many industrial species and has a history of safe use in the food industry [14]. There are few reports on lantibiotic synthesis in B. amyloliquefaciens. Production of mersacidin, a type-B lantibiotic, has been reported in B. amyloliquefaciens subsp. plantarum B6901-Y2 and B. amyloliquefaciens HIL Y-85,54728 (formerly Bacillus sp. HIL Y-85,54728) [7,15,16]. The biosynthetic cluster involved in mersacidin biosynthesis is composed of ten genes that span over 12.3 kb in the genome [6]. The biological mode of action of mersacidin is related to its ability to bind to the lipid II and thus to prevent peptidoglycan biosynthesis [7].

Recently, we have identified Bacillus amyloliquefaciens GA1 as a producer of a proteinaceous compound with potent antimicrobial activity toward the foodborne pathogen Listeria monocytogenes [17]. The failure of structural gene detection for all the genetically described bacteriocins from the Bacillus genus strongly suggests that this antimicrobial peptide, named amylosin, corresponds to a novel bacteriocin not described to date. Prior intensive characterization, amylosin was purified to test its ability to inhibit the growth of L. monocytogenes in poultry meat upon long-term storage [17]. In the present paper, we report on the biochemical characterization of that novel bacteriocin, the nucleotide sequence of the gene cluster involved in its biosynthesis and peculiar features on its inhibition spectrum and structural traits.

Results

Inhibition spectrum

The biological activity of the purified amylosin was characterized by determining the minimal inhibitory concentration (MIC) for an array of bacterial and fungal indicator strains. As shown in table 1, amylosin showed an antibacterial spectrum directed toward Gram-positive bacteria. Indeed, no growth inhibition was observed in our experimental conditions neither on both Basidiomycetes and Ascomycetes yeasts (i.e. Cryptococcus neoformans and Saccharomyces cerevisiae, respectively), nor on Gram-negative bacterium (i.e. Escherichia coli and Pseudomonas aeruginosa). By contrast, a significant inhibitory effect was observed for Enterococcus faecium with a MIC value of 0.1 µM and for Enterococcus faecalis in a lesser extent. Among the Bacillus genus, the opportunistic pathogen B. cereus that is a common cause of food poisoning was also found very sensitive with an MIC value of 0.2 µM. The growth of L. monocytogenes, another major food poisoning bacteria was also found sensitive to amylosin with an MIC value close 0.5 µM for the three clinical or food isolates tested, confirming thus previous observation [17]. S. aureus, including methicillin-resistant isolates (0.4 µM), together with S. epidermidis (2.8 µM), which are both opportunistic human pathogens, were also found sensitive to amylosin. For lactic acid bacteria such as Weissella sp. and Lactobacillus plantarum, only a weak or no growth inhibition effect was observed in our experimental conditions.

Amylosin susceptibility to proteases, heat and pH

Incubation of purified amylosin with pronase and proteinase K led to a strong decrease of the antimicrobial activity against the indicator strain Micrococcus luteus ATCC 9341 (Table 2). By contrast, no significant reduction of the amylosin biological activity against the indicator strain was observed upon heat treatments at different temperatures or incubation at various pH (Table 2). Indeed, incubation of purified amylosin at 100 ºC for 1 hour, led only to a 7% reduction of its biological activity whereas incubation in acidic (pH 2) and alkaline (pH9) condition led to an 11% and 7% antimicrobial activity reduction, respectively. These results were further confirmed by the comparison of the HPLC chromatograms corresponding to treated and non-treated amylosin samples (data not shown). This demonstrates that amylosin correspond to a heat and pH stable proteinaceous compound.

Gene cluster sequencing and characterization

In silico analysis of the 461.5 kb fragment of the B. amyloliquefaciens GA1 chromosome, obtained by partial shotgun sequencing [18], led to the identification of a 800 bp fragment encoding an amino acid sequence that exhibits 38 % and 33 % identity with the lantibiotic modification enzyme from
Table 1. Inhibition spectrum of amylolysin.

| Strains                        | Culture medium, Temperature (°C) | MIC (µM) |
|--------------------------------|----------------------------------|----------|
| Micrococcus luteus ATCC 9341   | BH, 37                           | 0.7      |
| Staphylococcus epidermis ATCC 1228 | BH, 37                           | 2.8      |
| Staphylococcus aureus ATCC 25923 | BH, 37                           | 2.8      |
| Staphylococcus aureus ATCC 43300* | BH, 37                           | 0.4      |
| Staphylococcus aureus RFB127*   | BH, 37                           | 1.4      |
| Enterococcus faecalis ATCC 29212 | BH, 37                           | 1.4      |
| Enterococcus faecalis RFB129*   | BH, 37                           | 0.7      |
| Enterococcus faecium RFB128*    | BH, 37                           | 0.1      |
| Listeria monocytogenes LMG 23905 | BH, 37                           | 0.4      |
| Listeria monocytogenes LMG 21263 | BH, 37                           | 0.5      |
| Listeria monocytogenes LM2234*  | BH, 37                           | 0.4      |
| Listeria innocua ATCC33090      | BH, 37                           | 0.7      |
| Listeria innocua RFB159*        | BH, 37                           | 0.7      |
| Listeria ivanovii RFB160        | BH, 37                           | 0.8      |
| Bacillus cereus RFB125*         | LB, 37                           | 0.2      |
| Bacillus subtilis ATCC 6633     | LB, 37                           | 1.4      |
| Bacillus megaterium RFB124      | LB, 37                           | 0.4      |
| Streptococcus agalactiae RFB141* | BH, 37                           | 2.8      |
| Weissella sp RFB139*            | MRS, 27                          | 2.8      |
| Lactobacillus plantarum RFB138* | MRS, 27                          | >2.8     |
| Escherichia coli RFB149*        | LB, 37                           | >2.8     |
| Pseudomonas aeruginosa RFB148*  | LB, 37                           | >2.8     |
| Cryptococcus neoformans IHEM3969 | YPD, 30                          | >2.8     |
| Saccharomyces cerevisiae RYF100  | YPD, 30                          | >2.8     |

* Methicillin resistant.

Table 2. Amylolysin stability.

| Factors       | Remaining activity (%) |
|---------------|------------------------|
| Protease      |                        |
| Control       | 100                    |
| Proteinase K  | 0                      |
| Pronase       | 20                     |
| PHa           |                        |
| 2             | 89                     |
| 3             | 98                     |
| 4             | 96                     |
| 5             | 99                     |
| 6             | 96                     |
| 7             | 100                    |
| 8             | 97                     |
| 9             | 93                     |
| Temperatureb  |                        |
| 25°C          | 100                    |
| 55°C          | 98                     |
| 65°C          | 99                     |
| 75°C          | 99                     |
| 100°C         | 93                     |

a Values were normalised as a percentage of the value obtained at physiological pH.
b Values were normalised as a percentage of the value obtained at 25°C.

Beside this, amIK and amIR, located upstream of amlA were found to encode proteins that present strong similarities with two-component regulatory proteins. AmIK shows 32 % of identity with C-terminal cytoplasmic domain of the Bacillus subtilis 168 histidine kinase ComP that acts as a membrane sensor of environmental signals. In this conserved domain, His^30^ and Asp^161^ are the autoprophosphorylated residue and the catalytic amino acid conserved in NisR, SpaR and ComP histidine kinases, respectively [23,24]. In addition, the conserved glycine rich stretch was found between amino acid 163 and 203 [13,24] (data not shown). AmIR exhibits a 46 % of identity with transcriptional regulators belonging to LuxR family (GI: 251795068). Indeed, in its N-terminal sequence, the characteristic K^3^ILxxDD^9^ and L^4^xxLD^9^ motifs (including catalytic residues Asp^9^ and Asp^20^) and the lysine residue (Lys^15^) of response regulators are found.

In the nucleic acid fragment obtained by IPCR, the amylolysin immunity and transport genes are missing. To identify these later, a BlastX search through prokaryotic protein databases was performed by using the 5.5 kb fragment as the query sequence. For amIK, amIR and amIM, a high identity (100% on nucleotide level) was found with sequences from Bacillus amyloliquefaciens IT45 annotated as sensor histidine kinase (GI: 363725376), LuxR family transcriptional regulator (GI: 363725375) and putative LanM like protein (GI: 363725373), respectively. To obtain the missing sequence of

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_Bacillus licheniformis_ ATCC 14580 (Genbank identifier (GI): 304557386) and _Bacillus halodurans_ C-125 (GI: 15613018), respectively. Further characterization of this locus led to the identification of different genes homologous to genes involved in lantibiotic biosynthesis. Indeed, from a 5.5 kb fragment obtained by inverse polymerase chain reaction (IPCR) [19], four complete ORFs were identified (Figure 1A). Of these, the so-called _amlA_ codes for a polypeptide of 60 residues that presents 35 % and 40 % of identity with the MrsA mersacidin peptide from _B. amyloliquefaciens_ HIL Y-85,54728 and _Bacillus pseudomycoides_ DSM 12442, respectively. A detailed analysis of _AmlA_ sequence revealed the presence of a GG sequence corresponding to the signal peptidase cleavage site of type-B lantibiotics (Figure 1B). The GxxxxTx(S/T)x(D/E)(C(3-10)xC motif present in all mersacidin and lacticin 481 like peptides was also observed together with the CxxTxC amino acid sequence known as essential for interactions with the peptidoglycan biosynthesis precursor lipid II [20,21]. Located downstream of _amlA_, _amlM_ encodes a putative 908 amino acids protein that exhibits 30 % of identity with the lantibiotic modifying enzyme LchM1 from _Bacillus licheniformis_ ATCC 14580. Multiple sequence alignment of _AmlM_ N-terminal domain (residues 64 to 364) with other homologous modification enzymes highlights the presence of the conserved motifs previously described for this type of enzymes (Figure 1C) [6,22].
the amylolysin biosynthetic gene cluster, primer walking was performed using *B. amyloliquefaciens* IT45 genome sequence as a template (GI: 423191475). This led to the identification of a 1.9 and 2.1 kb fragment upstream and downstream of *amlK* and *amlA*, respectively (Figure 1). Of these, three ORFs, designated as *amlT*, *amlF* and *amlE*, were identified and the deduced amino acid sequences are identical to those present in the *B. amyloliquefaciens* IT45 sequence and annotated as ABC transporter-like protein (GI: 363725372), efflux ABC transporter ATP-binding protein (GI: 363725380) and hypothetical protein KSO14349 (GI: 363725379), respectively. A search for specific motifs in these three amino acid sequences highlights the following similarities. The N-terminal part of *AmlT* belongs to C39 bacteriocin-processing peptidase superfamily [11]. More precisely, Cys<sup>19</sup>, His<sup>97</sup> and Asp<sup>113</sup> were identified as the putative catalytic amino acids conserved in these peptidases (data not shown) [25]. Beside this, the C-terminal part of *AmlT* sequence was found to exhibit similarities with ABC transporter superfamily, suggesting its role in amylolysin transport (data not shown). *AmlE* exhibits 36% identity with the membrane-bound part of a lantibiotic ABC transporter (GI: 260687109) from *Clostridium difficile* R20291, whereas *AmlF* shows 44% identity with MrsF (GI: 385266873) from *Bacillus* sp. 5B6, involved in lantibiotic self-protection. This latter contains a conserved domain of ABC transporter and belongs to the P-loop NTPase superfamily (data not shown) [26]. Therefore, it is likely that *AmlE* and *AmlF* could be involved in immunity mechanism with *AmlF* being the ATP-binding subunit and *AmlE* the efflux protein of an ABC transporter. The different *aml* gene sequences were deposited at GenBank under the accession number KC415250.1 (GI: 448918122).

**Disruption of *amlA* gene**

In order to correlate the antimicrobial activity of amylolysin to the putative lantibiotic gene cluster, the structural *amlA* gene was disrupted by insertion of a kanamycin resistance gene into *amlA* by double homologous recombination (Figure 1A). Culture supernatant of the resulting RFB137 insertion mutant (Table S1) was characterized by a loss of antibacterial activity
against *M. luteus* ATCC 9341 compared to that of the wild-type strain (Figure 2, insert). To further characterize the mutant phenotype, concentrated culture supernatant from RFB137 strain were analyzed by RP-HPLC and compared to that of the parental strain. As shown in Figure 2, the 21.5 min peak corresponding to amylolysin is lacking for RFB137 sample confirming that identified locus is involved in amylolysin biosynthesis.

**Structural characterization**

The presence of lanthionine, the structural characteristic trait of lantibiotics, was evidenced by LC-MS analysis. Amylolysin was first hydrolyzed in acidic conditions and OPA derivatized prior analysis (Figure 3 insert). In the resulting chromatogram, compounds eluted at retention time of 13.2 min in amylolysin hydrolysate and lanthionine standard samples, point out the presence of lanthionine [27]. Moreover, m/z signals at 315 deduced from the MS spectra confirm that amylolysin is a lantibiotic senso stricto (Figure 3, insert). Similar results were obtained with the well-characterized lantibiotic nisin (data not shown). Matrix-assisted laser desorption ionization time of flight mass spectrometry analyses (MALDI-TOF) of purified amylolysin allowed the determination of its molecular mass (Figure 3). Signal at m/z of 3317.6 obtained in negative mode of measurement highlighted a molecular mass of 3318.6 Da. The absence of this particular molecular mass in proteomic databases suggests that amylolysin could be a novel compound not described to date.

**Interaction with Lipid II**

*In silico* analysis of AmlA highlighted the C16TLTWEC11 sequence as a putative interaction site between amylolysin and the lipid II. In order to confirm this hypothesis, two experiments were designed. In the first one, a direct amylolysin-lipid II interaction was characterized while in the second the ability of amylolysin to inhibit the transglycosylation reaction involved in peptidoglycan synthesis was investigated [28]. In both experiments, interactions were analyzed by thin layer chromatography based on the utilization of [14C] lipid II. As shown in Figure 4A, the delay of migration of the amylolysin-lipid II complex compared to that of free lipid II traduced the direct interaction between the two partners. By contrast, inhibition of the transglycosylation reaction resulted in a not polymerized lipid II that migrates compared to polymerized form remaining at the origin (Figure 4B).

**Discussion**

The majority of the bacteriocins described to date are from lactic bacteria with nisin, used commercially in many countries as preservative in food products [29], being the best characterized compound. However, its sensitivity to proteases, its low solubility above pH 6 and emergence of nisin resistant strains, point out the need for alternative producer organisms [30]. In regards to their high level of protease production, *Bacillus* sp. has been considered as interesting alternative source for antimicrobial peptides with increased protease tolerance. In our laboratory, *B. amyloliquefaciens* GA1 has been characterized for its ability to produce antimicrobial metabolites [18]. Beside polyketides (PKs) and non-ribosomally synthesized peptides (NRP), GA1 was found to produce amylolysin, a bacteriocin that is less sensitive to meat proteases than nisin [17].

The prerequisites for the molecular characterization of amylolysin was to scale-up and to adapt the production process previously reported [17]. In addition, a GA1 derivative RFB136 mutant, unable to produce NRPs and PKs, was used...
to prevent contaminations by other antimicrobial compounds during the purification process. The thermo-stability and pH tolerance together with the structural characterization by LC-MS demonstrated that amylolysin belongs to the class I bacteriocin family. Moreover, the presence of one unique AmlM modification enzyme and an AmlT transport protein with a peptidase-like associated domain suggests that amylolysin belongs to the type-B lantibiotics. The

Figure 3. MALDI-TOF MS and LC-MS analysis of amylolysin. Mass spectrum of purified amylolysin sample was recorded in negative mode. Insert: LC-MS chromatograms of commercial lanthionine standard and amylolysin hydrolysate. Intensity (%, Y-scale) was recorded by setting the SQD mass analyzer on the specific mass of lanthionine (315 Da).

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Figure 4. Interaction with lipid. (A) Direct interaction of amylolysin with lipid II. Both compounds were incubated in DMSO/1-octanol mixture (60/40 v/v) for 1 hour at room temperature before being subjected to TCL using a butanol/acetic acid/pyridine/water mixture (15/3/12/10, v/v/v/v) as mobile phase. (B) Inhibition of the transglycosylation reaction. Amylolysin, lipid II and glycosyltransferase were incubated for one hour before being subjected to TLC using a mixture of methanol/chloroform/ammoniac/water (88/48/10/1, v/v/v/v) as mobile phase. [14C] lipid II was detected with a Molecular Imager FX system. Direction of solvent migration during TLC is indicated by the arrow.

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absence of significant similarity between AmlA and bacteriocins reported in BACTIBASE and Antimicrobial Peptide Database, points out that amylolysin corresponds to a novel lantibiotic not described to date. Reports on type-B lantibiotic from *B. amyloliquefaciens* are scarce. So far, a gene cluster for lantibiotic synthesis has been reported in *B. amyloliquefaciens* subsp. *plantarum* strain YAU B9601-Y2 [31]. Beside this, mersacidin production has been reported recently in *B. amyloliquefaciens* subsp. *plantarum* B6901-Y2 and *B. amyloliquefaciens* HIL Y-85,54728 [7,15,16]. The biosynthetic cluster consists in a structural gene *mrsA*, together with gene *mrsM*, involved in posttranslational modifications of the mersacidin prepeptide; *mrsT*, coding for a transporter with associated protease domain. The immunity genes *mrsF, mrsE* and *mrsG* and the regulatory genes *mrsR1, mrsR2* and *mrsK2*, respectively, are also present. The two-component regulatory system MrsR2/MrsK2 is mainly involved in immunity and self-induction of mersacidin biosynthesis [32] whereas Mrs1 was found essential for mersacidin production [33]. The presence of genes involved in mersacidin self-protection has been also detected in the genome of *B. amyloliquefaciens* FZB42 [34]. Despite that strain FZB42 is unable to produce mersacidin, it has been used recently as a host cell for the transfer of the mersacidin biosynthesis genes from *B. amyloliquefaciens* HIL Y-85,54728 [15].

A close inspection of *amiA* sequence showed that it codes for a 60 residues prepeptide with a specific GGGG motif. Type-B lantibiotics are known to possess leader sequences ranging from 15 to 40 amino acids that are processed within G(G/A/S)(X)XX motif. Thus, the GGGG sequence found in AmlA is the best candidate for processing site of the signal peptide. However, the presence of four glycine residues allows three possibilities for this processing (i.e. after glycine at position 27, 28 and 29, respectively). MALDI-TOF measurement has highlighted a mature AmlA peptide of 3318 Da that is consistent with a cleavage by AmM after G29. Thioether bridging patterns have been determined for several type-B lantibiotics including lacticin 3147 [35] and haloduracin [21]. Off these, the conserved CtxTxEC motif was found involved in the formation of one Lan residue and two MeLan subunits with LanF being the ATP-binding domain [39]. In lacticin 3147 producer strains, it has been shown that LtnE and LtnF play an important role in immunity mechanism even at a greater degree than LtnI. From this viewpoint, amylolysin gene cluster is also atypical. Only two genes, namely AmlF and AmlE, have been detected in GA1 strain suggesting that immunity to amylolysin is ensured by a two-component ABC transporter. The immunity mechanism is therefore somewhat different from that found in *B. amyloliquefaciens* for mersacidin self-protection [15,16].

This first characterization of amylolysin highlights some divergences with the one-component type-B lantibiotic usually produced by *Bacillus* sp. However, structural as well as mechanistic data are still lacking to deeply characterize this promising lantibiotics. The fact that amylolysin interacts with lipid II, is certainly a first element to explain its biological mode of action. Experiments are in progress to get those lacking information.

### Materials and Methods

#### Bacterial strains, culture media and general genetic techniques

*Bacillus amyloliquefaciens* GA1 was used for amylolysin production [17]. The different bacterial and hyphal isolates used as indicator strains are listed in table 1. *Escherichia coli* DH5α (Promega, Madison, WI, USA) was used for transformation and amplification of recombinant plasmid DNA according standard procedures [40]. *B. amyloliquefaciens* GA1 was transformed as described elsewhere [41]. Luria-Bertani (LB), brain-heart (BH), de Man-Rogosa-Sharpe (MRS), and YPD media were of commercial origin (Becton-Dickinson, Le pont de Chaix, France). Microbial growth was monitored by optical density measurement at 600 nm (OD<sub>600</sub>). Genomic DNA was purified using the Wizard genomic purification kit (Promega). PCR amplifications were performed with Fidelity Taq polymerase (USB Corporation, Cleveland, OH, USA) and amplified fragments were purified with the Qiagen purification kit (Hilden, Germany). PCR fragments were cloned into plasmid T-easy vector prior sequencing at GIGA Genomics Facilities (University of Liège, Liège, Belgium). Primers used for PCR amplifications, plasmids and bacterial strains are listed in Table S1. For similarity searches, the FASTA program [42] was used to scan UniProtKB/Swiss-Prot and UniProtKB/TrEMBL databases. Sequence alignments were generated using ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and manually adjusted in Genedoc (http://www.nrbsc.org/gfx/genedoc/) to correct obvious mism pairings. Predictions terminators were performed using ARNold program [43] whereas BPRom program was used for prediction of putative promoter elements (http://linux1.softberry.com/all.htm). Blasts analysis were performed at the NCBI website (http://blast.ncbi.nlm.nih.gov/Blast.cgi).
Amylolyisin production, purification and quantification

Large-scale production of amylolyisin was performed in a 80-liters Bioflo 5000 bioreactor (New Brunswick) in a working volume of 60 L of LB medium with strain RFB136 (a derivative of strain GA1 unable to produce lipopeptides, see Text S1). Cultures were conducted at 37 °C for 10 h at a stirring speed of 200 rpm and an aeration flow of 1 VVM (volume of air per volume of medium per minute). Cell-free supernatant was obtained by cross flow filtration using a hollow fiber cartridge (0.45 μm, 8400 cm², GE Healthcare) according to the manufacturer’s recommendations. Amylolyisin was purified in a three steps procedure. Firstly, the antimicrobial peptide was extracted from the culture supernatant using an Amberlite XAD-16 resin (Sigma-Aldrich) and concentrated by rotary evaporation. Amylolyisin was subsequently purified in two steps of reverse-phase RP-HPLC using a VP Nucleosil column (25 x 10 mm, 7 μm packing, Macherey-Nagel) and a Nucleodur C18 column (50 x 4.6 mm, 5 μm packing, Macherey-Nagel) at a flow rate of 2.5 and 1 ml min⁻¹ of an acetonitrile/H₂O mixture (41/59, v/v), respectively. Amylolyisin identification and quantification were performed by RP-HPLC as described previously [17].

Effect of enzyme, pH and temperature on amylolyisin activity

To evaluate sensitivity to proteases, purified amylolyisin samples were quantified by RP-HPLC after 24h of incubation at 37°C with proteinase K and pronase (10 μg/ml, Sigma-Aldrich). Heat stability was evaluated after incubation for 60 min at various temperatures (25, 55, 65, 75 and 100 °C) whereas pH sensitivity was evaluated after a 1h-incubation in 200 mM HCl/KCl buffer (pH 2-3), acetate buffer (pH 4-6) and Tris buffer (pH 7-9). After those treatments, the remaining amylolyisin activity was determined using the agar diffusion assays as described elsewhere, using Micrococcus luteus ATCC 9341 as an indicator strain.

Minimal inhibitory concentration determination

Minimal inhibitory concentration (MIC) was determined for an array of bacterial and yeast strains by a microdilution method following the Clinical and Laboratory Standard Institute (CLSI) recommended procedure [44]. Briefly, the different indicator strains were aerobically grown in 24-wells culture plates in the presence of purified amylolyisin at concentration ranging from 0.1 to 2.8 μM in the appropriate medium and temperature conditions as stipulated in table 1. Each well was seeded at an OD₆₀₀ of 0.1. After 24 h of incubation, cell growth was estimated based on OD₆₀₀ measurements. The MIC values were defined as the lowest amylolyisin concentration that is able to inhibit cell growth. Each experiment was performed in triplicate.

Characterization of amylolyisin biosynthesis genes

Identification of the genomic locus involved in amylolyisin biosynthesis was performed by inverse polymerase chain reaction (IPCR) [19]. Briefly, 5 μg of purified genomic DNA of B. amyloliquefaciens GA1 were first digested with restriction enzyme PstI (1U/μg for 2h). The digested DNA was then purified before being self-ligated using T4 DNA ligase (Promega). PCR amplification was then performed using primer pair RFO142/RFO143 and the self-ligation mixture as a template with an elongation time of 10 min. The resulting PCR fragment was then cloned into pGEM-T-easy vector and sequenced by primer walking. The resulting sequence was then used for Blast search for further characterization of the amylolyisin locus.

Disruption of amlA

Disruption of amlA was performed as described previously [45]. Briefly, the 407 and 523 bp P and T fragment consisting of part of the 3’ and 5’ amlA ORF were PCR-amplified using primer pairs P2B4sens/P2B4rev and T2B4sens/T2B4rev, respectively, and B. amyloliquefaciens GA1 genomic DNA as a template. Primers P2B4rev and T2B4sens contain the rare meganuclease I-SceI recognition sequence. P-I-SceI and I-SceI-T fragments were then pooled and used as a template for amplification of the P-I-SceI-T cassette with primers P2B4sens and P2B4rev. The resulting fragment was then cloned into pGEM-T easy vector to generate RFPA1. The 1.6 kb fragment encoding a kanamycin resistance gene was rescued from RFP104 [41] by I-SceI digestion and subcloned into RFPA1 at the corresponding restriction site to yield RFPA2. The amlA disruption cassette was finally obtained by PCR amplification with primers pair P2B4sens/T2B4rev and RFPA2 as a template and used, after purification, to transform B. amyloliquefaciens RFB136. Transformants were selected on LB-kanamycin plates (10 μg/ml). Integration by double-crossing event in the RFB136 strain was verified by analytical PCR using primer pair P2B4sens/T2B4rev. The amlA-disrupted strain was denominated RFB137.

Mass spectrometry

The molecular mass of the purified amylolyisin was determined by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry using a 4700 Proteomics analyzer (Applied Biosystem). Purified amylolyisin was mixed with an equal volume of o-cyanohydroxycinnamic acid solution (10 mg/ml) and spotted onto the MALDI plate. The analyzer was used at an acceleration voltage of 20 kV. Samples were measured in the reflectron mode. Mass spectral databases and proteomic tools were MassBank (http://www.massbank.jp) and Prospector (http://prospector.ucsf.edu/).

The presence of the modified amino acid lanthionine in amylolyisin was demonstrated by RP-HPLC coupled with single quad mass spectrometer (HPLC Waters Alliance 2695/diode array detector, coupled with Waters SQD mass analyzer) on a X-terra MS column (150 x 2.1 mm, 3.5 μm packing, Waters) after peptide hydrolysis (HCl 6 M, for 4 h à 145°C) and derivatization with o-phthaldialdehyde (OPA) (Agilent Technology) as described elsewhere [46]. Elution was performed at a constant flow rate of 0.25 ml min⁻¹ and at 40°C, with a gradient of acetonitrile (solvent B) in water acidified with 0.1% formic acid (solvent A) as follows: 0-2 min, 0% B; 2-7 min, 0 to 10% B; 7-17 min, 10 to 15% B; 17-19 min, 15 to 95%
B). Compounds were first identified on the basis of their retention times compared with a commercial lantionine standard (Sigma-Aldrich). The identity of lantionine was subsequently confirmed on the basis of the masses detected in the SGD by setting electrospray ionization (positive ion) conditions in the MS as source temperature, 130°C; desolvation temperature, 250°C; nitrogen flow, 500 l h⁻¹; cone voltage, 50 V.

Interaction with Lipid II

Radiolabelled UDP-MurNac-pentapeptide, thereafter [¹⁴C] lipid II, was prepared in vitro using E. coli K12 purified membrane fraction: UDP-GlcNac and UDP-MurNac-L-Ala-D-Glu-meso-DAP-D-[¹⁴C]Ala-D-[¹⁴C]Ala and subsequently purified as previously described [28]. Inhibition of glycosyl transferase reaction was performed as described elsewhere [47] with some modifications. Briefly, [¹⁴C] lipid II (2.5µM; 0.125µCi nmol⁻¹), amylolysin (0.2 µM), His-tag PBP1b γ from E. coli (15 nM) were incubated in 25mM Tris-HCl (pH7.5), 0.5 % decy PEG, 10 mM MgCl₂, 12 % 1-octanol, 25 % DMSO for 1 hour at 30 °C. The reaction products were separated by thin layer chromatography (TLC) on silica plate (SilG, 250 µM thickness, Macherey-Nagel, Düren, Germany) using a mixture of methanol/chloroform/ammoniac/water (88/48/10/1, v/v/v/v) as mobile phase. For interaction experiment, [¹⁴C] lipid II (2.5 µM; 0.125µCi nmol⁻¹) and amylolysin (0.2 µM) were incubated in DMSO/1-octanol mixture (60/40 v/v) for 1 hour at room temperature before being subjected to TCL using a butanol/acetic acid/pyridine/water mixture (15/3/12/10, v/v/v/v) as mobile phase. The radioactive compounds were detected and analyzed with a Molecular Imager FX system (Biorad Laboratories).

Supporting Information

Figure S1. Sequence alignment of the N-terminus of type B lantibiotic modification enzymes.

Table S1. Strains and plasmids used in this study.

Text S1. Construction of strain RFB136.

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Author Contributions

Conceived and designed the experiments: AAA MT PF. Performed the experiments: AAA MO BD MT PF. Analyzed the data: AAA MO BD BJ PT. Contributed reagents/materials/analysis tools: MO BD MT PF. Wrote the manuscript: AAA BJ PF.

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