Identification and characterization of andalusicin: N-terminally dimethylated class III lantibiotic from Bacillus thuringiensis sv. andalousiensis

Highlights
Type III lanthipeptide andalusicin A inhibits the growth of Gram-positive bacteria
Andalusicin A has an unusual pastor’s crook structure
Genes encoding methyltransferases are frequently found in andalusicin-like BGCs
N-terminal methylation is necessary for andalusicin A biological activity
Identification and characterization of andalusicin: N-terminally dimethylated class III lantibiotic from *Bacillus thuringiensis* sv. *andalousiensis*

Anastasiia Grigoreva,1,2 Julia Andreeva,1,2 Dmitry Bikmetov,3,4 Anastasiia Rusanova,2 Marina Serebryakova,2,5 Andrea Hernandez Garcia,6 Darya Slonova,1 Satish K. Nair,6 Guy Lippens,7 Konstantin Severinov,1,3,4,8,9,* and Svetlana Dubiley1,2,*

**SUMMARY**

Lanthipeptides, ribosomally synthesized and post-translationally modified peptides (RiPPs), can be divided into five classes based on their structures and biosynthetic pathways. Class I and II lanthipeptides have been well characterized, whereas less is known about members of the other three classes. Here, we describe a new family of class III lanthipeptides from *Firmicutes*. Members of the family are distinguished by the presence of a single carboxy-terminal labionin. We identified and characterized andalusicin, a representative of this family. Andalusicin bears two methyl groups at the α-amino terminus, a post-translational modification that has not previously been identified in class III lanthipeptides. Mature andalusicin A shows bioactivity against various Gram-positive bacteria, an activity that is highly dependent on the α-N dimethylation.

**INTRODUCTION**

Lanthipeptides represent the largest known family of ribosomally synthesized and post-translationally modified peptides (RiPPs). They are synthesized as linear precursor peptides by the ribosome and then undergo post-translational modifications (PTMs) (Arnison et al., 2013; Montalbán-López et al., 2021). The PTMs are localized in the core part of the precursor peptide, whereas the leader sequence recruits the modification enzymes. After the modifications are installed, the leader is cleaved off during (or sometimes after) export from the producing cell (Oman and van der Donk, 2010). Genes required for biosynthesis of bacterial RiPPs usually form compact biosynthetic gene clusters (BGCs). Lanthipeptide BGCs contain gene(s) encoding the precursor peptide(s) and enzymes required for post-translational introduction of the family-defining lanthionine cross-link in the precursor (Figure 1). In addition, some lanthipeptide BGCs contain accessory genes coding for export pumps and/or self-immunity mechanisms (Stein et al., 2003), leader peptides (Lagedroste et al., 2017), and various tailoring enzymes responsible for secondary PTMs (Acedo et al., 2019; Iorio et al., 2014; Wiebach et al., 2018).

The lanthionine (α-thioether linkage) is installed in two steps, which are catalyzed by a multifunctional lanthionine synthetase (in class II–IV lanthipeptides), the sequential action of dehydratase and cyclase (in class I lanthipeptides) (Repka et al., 2017), or putative lyase, kinase, and cyclase (in class V) (Xu et al., 2020). Select Ser or Thr residues of the precursor are dehydrated to dehydroalanine (Dha)/dehydrobutyrine (Dhb). Next, a thiol group of a Cys is added to the double bond in a 1,4 conjugate addition reaction to form either a lanthionine (Lan) or a methyl-lanthionine (MeLan), respectively (Knerr and van der Donk, 2012). C-terminal lanthionine and MeLan can undergo decarboxamidation resulting in (2-amino-2-oxvalyl)-cysteine (AviCys) and 2-amino-3-methyl-cysteine (AviMeCys), respectively (Ortega et al., 2017; Schneider et al., 2000). In some cases lanthipeptides contain more complex labionin (Lab)/methyllabionin (MeLab) structures, which form upon the dehydration and macrocyclization of three residues, Ser/Ser/Cys or Ser/Thr/Cys, respectively (Figure 1) (Meindl et al., 2010).

Class III–V lanthipeptides are the least studied, and only a handful of representatives of either of these classes are known. Class III–IV lanthipeptides are synthetized by a multifunctional lanthionine synthetase composed of three domains (Figure 1): the central kinase domain that activates Ser and Thr residues through phosphorylation; the N-terminal lyase domain, responsible for phosphate elimination; and the
C-terminal cyclization domain (LanC) that catalyzes the conjugate addition (Hegemann et al., 2019). The latter domain discriminates class III (LanKC) and IV (LanL) enzymes, as LanKC C-terminal domains do not contain the characteristic zinc-binding residues conserved in other cyclases (Wang and van der Donk, 2012). The unique feature of LanKC enzymes is the ability to install labionin/methyllabionin structures; however, the exact mechanism of this process remains unknown (Hegemann and Süssmuth, 2020).

Here, we report the identification and functional characterization of a novel lanthipeptide biosynthetic gene cluster from Bacillus thuringiensis sv. andaloussiensis NRRL B23139 and structural characterization.
of its labionin-containing product, which we named andalusicin. We identified a methyltransferase responsible for \(\alpha\)-N-methylation of andalusicin, a tailoring modification previously unknown in class III lanthipeptides. We show that this \(\alpha\)-N-methylation is required for antibacterial activity of andalusicin.

**RESULTS**

**A novel lantibiotic biosynthetic gene cluster in the *Bacillus thuringiensis* NRRL B23139 genome**

During screening of laboratory collection of microorganisms for bioactivity, we observed that *B. thuringiensis* sv. *andalousiensis* NRRL B23139 inhibits growth of *B. cereus* ATCC 4342 (Figure 2A). To identify the compound responsible for antimicrobial activity, the cellular extract of *B. thuringiensis* sv. *andalousiensis* NRRL B23139 was fractionated using reverse-phase high-performance liquid chromatography (HPLC) (Figures S1A and S1B). MALDI-TOF-mass spectrometric (MS) analysis of the active fraction revealed a prominent [M + H]\(^+\) ion at m/z 2,259.1 (Figure S1B), which was also present in the starting extract (Figure 2B). MALDI-TOF-tandem MS (MS/MS) showed that the corresponding protonated molecule is a heavily modified peptide likely containing a number of dehydrobutyrines (Dhb), arising from dehydration of threonine residues (Figure 2C). Analysis of fragmentation spectra suggested that the bioactive compound may be a RiPP with its precursor a peptide containing an (I/L)TTTWTVTTTGVWA sequence.

We sequenced, assembled, and annotated the genome of *B. thuringiensis* sv. *andalousiensis* NRRL B23139 (GenBank: NZ_CP035727.2). Using the predicted partial sequence of the bioactive peptide as a query for
BlastP search, we identified a compact locus containing seven short open reading frames (ORFs), whose products matched the predicted partial sequence of the bioactive RiPP. Five of the seven genes code for identical 45-amino acid-long peptides (AncA1-2, AncA4-6), whereas two genes encode peptides harboring an alanine to valine substitution in the putative leader (AncA3) and in the core (AncA7) parts, respectively (Figure 2D). A gene encoding a class III lanthionine synthetase is located upstream of the peptide-coding locus. We therefore hypothesized that in addition to dehydrated residues, the identified bioactive compound may contain a lanthionine cross-link. The presence of the LxLQ motif characteristic for class III/IV lanthipeptides (Müller et al., 2011) in the leader part of the AncA peptides supports this hypothesis. Since expected C-terminal ions are absent from the MALDI-TOF-MS/MS spectra in the Figure 2C, a lanthionine could be formed by the C-terminal Cys23 and dehydrated Thr18, Ser17, or Ser20 residues.

Assuming that all Ser and Thr residues in the core parts of AncA peptides are modified and that the 22-amino acid long N-terminal leader is cleaved off, the calculated m/z values of protonated mature AncA1-6 should be 2,231.0. However, the observed mass of produced bioactive compound is higher by 28 Da. Careful inspection of mass spectra of the HPLC fractions identified a [M + H]+ ion at m/z 2,231.0. No biological activity was evident in fractions containing this compound (Figures S1A and S1C). Although the measured mass of this compound matches the calculated mass of fully modified AncA1-6 peptides, its fragmentation pattern corresponds to AncA7 (Figure S1D). Similar to the major compound, the additional 28 Da were assigned to the N-terminal dipeptide of AncA7-based compound. We named the major compound produced from AncA1-6 peptides andalusicin A, and the minor compound produced from AncA7, andaluscin B.

Andaluscin A is dimethylated

The ancMT gene encoding a putative S-adenosyl methionine (SAM)-dependent methyltransferase is located immediately downstream of the andalusicin peptide-coding locus. We speculated that ancMT is part of the andalusicin BGC and that its product may be responsible for dimethylation of andaluscin A. To test this hypothesis, we expressed subsets of anc genes in a heterologous host. When a minimal set of two genes, ancKC-ancA1, under control of an inducible promoter was introduced in B. subtilis 168 strain, a modified peptide (an [M + H]+ ion at m/z 2,231.0) accumulated in induced cells. Its molecular mass and fragmentation spectrum matched those of andalusicin A but for the extra 28 Da (Figures 3A, upper panel, and 3B). When the ancKC-ancA1 genes were introduced together with ancMT, a 28-Da shift of the modified peptide appeared (Figure 3A) and the MALDI fragmentation spectrum of the [M + H]+ ion at m/z 2,231.0 accumulated in induced cells. A gene encoding a putative S-adenosyl methionine (SAM)-dependent methyltransferase is located immediately downstream of the andalusicin peptide-coding locus. We speculated that ancMT is part of the andalusicin BGC and that its product may be responsible for dimethylation of andaluscin A. To test this hypothesis, we expressed subsets of anc genes in a heterologous host. When a minimal set of two genes, ancKC-ancA1, under control of an inducible promoter was introduced in B. subtilis 168 strain, a modified peptide (an [M + H]+ ion at m/z 2,231.0) accumulated in induced cells. Its molecular mass and fragmentation spectrum matched those of andalusicin A but for the extra 28 Da (Figures 3A, upper panel, and 3B). When the ancKC-ancA1 genes were introduced together with ancMT, a 28-Da shift of the modified peptide appeared (Figure 3A) and the MALDI fragmentation spectrum of the [M + H]+ ion at m/z 2,231.0 was identical to that obtained for andalusicin A secreted by the native producer. These results confirm that methyltransferase AncMT is responsible for the addition of two methyl groups to andalusicin. The methylation reaction was also reconstructed in vitro using purified desmethyl andaluscin A, recombinant AncMT, and SAM as a methyl donor. As shown in Figure 3C, AncMT successfully converted desmethyl andaluscin A ([M + H]+ ion at m/z 2,231.0) to its double-methylated form ([M + H]+ ion at m/z 2,259.1). The fragmentation spectrum of this in vitro reaction product was identical to that of andaluscin A secreted by the native producer.

Andaluscin A showed potent antimicrobial activity against B. cereus ATCC 4342 in the broth dilution assay with a minimal inhibitory concentration of 16.2 μg/mL, which is comparable to the reported activity of nisin (10 μg/mL) (Pol and Smid, 1999). To test the biological effect of dimethylation, we compared antimicrobial activities of dimethylated and desmethyl andaluscin A. Dimethylated andaluscin A was active against all Gram-positive bacteria tested, with closely related Bacillus strains being the most sensitive (Table S1). In the absence of methylation, antimicrobial activity against most bacteria tested was abolished. Weak growth inhibition by desmethyl andaluscin A was only observed for B. cereus ATCC 14579. We conclude that dimethylation dramatically increases the bioactivity of andaluscin A.

To get an insight into the mechanism of antimicrobial activity of andaluscin A, we monitored membrane integrity of B. cereus cells treated with andaluscin by staining with propidium iodide (Boulos et al., 1999). As expected, treatment with proton ionophore carbonyl cyanide 3-chlorophenylhydrazone (CCCP) did not result in cell lysis or propidium iodide accumulation inside the cells (Figure 4 and Table S2). In contrast, the addition of nisin caused intracellular accumulation of propidium iodide. Treatment with andaluscin had a similar effect (Figure 4 and Table S2). Nisin binds cell-wall precursor lipid II (Hsu et al., 2004) and induces large pore formation (Wiedemann et al., 2004) and lipid loss in the membrane.
of sensitive cells (Prince et al., 2016). Although both andalusicin and nisin induced membrane permeabilization for propidium iodide, their actual mechanism of pore formation might differ.

**Structural analysis of andalusicin A**

Bioinformatics analysis shows that AncKC is a member of class III lanthionine synthetases. These enzymes are able to install both lanthionine and labionin modifications, the latter being a unique cross-link present in only a handful of lanthipeptides (Hegemann and Süssmuth, 2020). As labionin/lanthionine cross-link formation does not change the net mass of the fully dehydrated peptide, MS alone cannot reveal the nature of the macrocycle. Susceptibility of the peptide to further chemical modifications provides indirect evidence of lanthipeptide cross-link structure. Under mild alkaline conditions, substituted thiols convert the Dha and Dhb residues to S-alkylated cysteine and \( \beta \)-methylcysteine, respectively, while leaving the lanthionine- or labionin-forming serine and threonine residues intact (Lohans and Vederas, 2014). The reaction with \( \beta \)-mercaptoethanol results in a 78 Da increase per Dha/Dhb residue. MS analysis of \( \beta \)-mercaptoethanol-treated andalusicin A revealed a 702-Da shift, which corresponds to alkylation of nine Dha/Dhb residues (Figures S2A–S2C). This suggests that 9 of 11 dehydrated serine and threonine residues in andalusicin A exist as Dha/Dhb, whereas the remaining two Dha/Dhb residues may be involved in a labionin cross-link.

Treatment of lantibiotics with nickel boride leads to peptide linearization through desulfurization of lanthionine cross-link (Martin et al., 2004). Dha and Dhb are converted, respectively, to alanine and \( \alpha \)-amino-butyric acid (Abu), whereas lanthionine is converted to two Ala residues that become susceptible to fragmentation during tandem MS. However, the fragmentation pattern of andalusicin A treated with nickel boride lacked product ion peaks in the Ser17–Ser20 region (Figures S2D and S2E). This could be the case if andalusicin contained a labionin in which the thioether bridge between residues 20 and 23 was desulfurized, but the carbocycle-forming residues 17 and 20 remained intact.
Solubility of andalusicin A in deuterated DMSO proved satisfactory to obtain high-resolution NMR spectra. Although the presence of 8 Dhb residues in the N-terminal part of the peptide hampered complete NMR characterization, we could assign all signals of amino acids with Hα protons based on combined homonuclear Total Correlation Spectroscopy (TOCSY) and Nuclear Overhauser Spectroscopy (NOESY) experiments complemented with 1H-13C Heteronuclear Single Quantum Correlation (HSQC) and Heteronuclear Multiple Bond Correlation (HMBC) spectra. As the only amino acid devoid of an amide proton, we identified the N-terminal Ala as the peak at 3.12/66.8 ppm for its 1Hα/13Cα group and 1.11/16.08 ppm for its methyl moiety (Figure 5A). Importantly, the random coil value for Ala 13Cα is at 50.5 ppm (Wishart et al., 1995), but the 16 ppm downfield shift we observe is compatible with the 13C chemical shift perturbation of 17 ppm observed in Lys side chain 13Cε upon dimethylation (Theillet et al., 2012). Furthermore, exploiting the long-range connectivity in the HMBC spectrum, we observed a distinct cross-peak between the Ala 1Hα/13Cα correlation and an intense peak at 2.226/43.4 ppm, whereby the latter values coincide with the chemical shift values for N-linked methyl groups (Figure 5A). These combined results confirm that the N-terminal Ala in andalusicin carries two methyl groups.

As for the labionin core, we exploited the combined NOESY/TOCSY spectra to identify characteristic NOE peaks. Starting from Gly in position 13, the full NMR assignment could be obtained on the basis of combined TOCSY and NOESY spectra (Table S3). Importantly, we observed a clear NOE contact between the Hα and Hβ protons of the residue in position 16 and the amide proton of residue in position 20. Together with the NOE contact between the α proton of the central residue in position 20 and the amide proton of residue 23 (Figure 5B), this confirms the constrained architecture of the labionin moiety. The physicochemical properties of andalusicin A are summarized in Figure 6.

Mutational analysis of the andalusicin precursor
To test substrate requirements of AncKC, we conducted a mutational analysis of ancA1 using B. subtilis harboring plasmid-borne ancKC-ancA1 genes. To check if a lanthionine ring can be formed instead of labionin, a mutation resulting in Ser20Ala substitution was introduced into ancA1. This substitution did not result in the MS/MS fragmentation pattern change indicating that the lanthionine ring was still formed in the mutant peptide (Figures S3A and S3B). Alanine substitution of Ser17 resulted in a short lanthionine ring, as evidenced by an MS/MS fragmentation pattern that lacked fragmentation peaks between Ser20 and Cys23 (Figures S3A and S3C). Unexpectedly, no mature products of the double S17A/S20A AncA1 mutant were detected, suggesting that Dhb18 or any other preceding Dhb cannot serve as an acceptor for the methyl-lanthionine cross-link. Taken together, our data indicate that AncKC can catalyze independent nucleophilic attack by AncA1 Cys23 on either one of the dehydrated serine residues, Dha17 or Dha20. To check if lanthionine-containing Ser20Ala mutant can be a substrate for AncMT, B. subtilis was transformed with a plasmid harboring the ancKC-ancA1 (S20A)-ancMT operon. As expected, N-terminally dimethylated lanthionine-containing andalusicin was produced (Figure 5D). Unfortunately, the efficiency of production was not sufficient for antibacterial activity analysis.

BGCs encoding class III lanthipeptides in Firmicutes
Recent genome mining of prokaryotic genomes revealed that putative BGCs encoding class III lanthipeptide biosynthesis encompass about a quarter of the entire lanthipeptide landscape (Walker et al., 2020).
Despite their abundance, class III lanthipeptides remain largely unexplored. As andalusicin is the first characterized labionin-containing lantibiotic from Firmicutes, we decided to get a closer look at its homologs. First, we built a sequence similarity network of the LanKC enzymes. As is shown in Figure 7A, at the BLAST score cutoff of 130, five major groups of class III LanKC were identified. Two groups (I and V) comprise solely actinobacterial sequences, groups II and IV contain predominantly firmicutal sequences, while group III has members from different phyla. Andalusicin AncKC belongs to group II. To further evaluate the diversity within this group, we built a phylogenetic tree of lanthionine cyclase domains (LanC), inspected proteins encoded in close proximity to lanKC genes, and analyzed ORFs encoding putative precursor peptides (Figure 7B and Table S4).

Figure 5. NMR spectra of andalusicin and the structure of C-terminal labionin cross-link
(A) 1H-13C HSQC (blue), HSQC-DIPSI (green), and HMBC (magenta) spectra of the andalusicin peptide in DMSO. The signals of the N-terminal Ala are circled in red, whereas the long-range connectivities with the N-terminal methyl resonance are circled in magenta.
(B) NOESY (red) and TOCSY (blue) spectra of the peptide in the fingerprint region. The distinct nuclear Overhauser effect (NOE) cross peaks for the residue in position 20 to both the residues in position 20 (red circles) and position 23 (magenta circle) confirm its central position in the labionin architecture.
The numbers of genes coding for predicted precursor peptides ranged from one to as many as 13, with a mean number of 2.8 and a median of 2. It is worth noting that if multiple genes for precursor peptides are present in a BGC, they encode peptides with identical or almost identical sequences. However, in some cases, predicted precursor peptides differ significantly (e.g., in *Curtobacterium* sp. MCBA15_003 or *Alkalihalobacillus marmarensis* DSM 21297 in Table S4), implying that some class III lanthipeptide BGCs may encode two-component lanthipeptides (McClerren et al., 2006).

We identified conserved motifs in predicted precursor sequences using MEME software (Bailey et al., 2009). As expected, the class III leader peptide motif VLXLQ (motif 1 in Figure 7C) and labionin motifs S(X)2S(X)2C or S(X)3S(X)2C were found (motifs 3 and 5, Figure 7C). Almost half of the precursors contained an extended tandem labionin motif (motif 2 in Figure 7C). We also identified an additional motif enriched in threonine residues (Figure 7C, motif 4) previously noted by (Walker et al., 2020). Mapping of predicted precursor peptide motif architectures to the LanC phylogenetic tree revealed, remarkably, that precursors with different motif architectures are associated with distinct clades. Precursors presumably containing two labionin macrocycles, either extended tandem motif 2 or various combinations of motifs 3 and 5, are associated with the basal clades of LanKC enzymes. A large group of precursors peptides contain the threonine-rich motif 4 followed by a single C-terminal labionin motif 3 or 5. These precursors are exclusively associated with a relatively recent LanKC clade (Figure 7B). As this clade contains AncKC characterized in this work, we will refer to it as “AncKC-like” and the peptides associated with this clade as “andalusicin-like.”

To establish the set of genes involved in firmicetal class III lanthipeptide production, we analyzed genes frequently found in the genomic neighborhood of *lanKC* genes (Table S4). In actinobacterial class III lanthipeptides, the leader peptide removal can be performed either by a bifunctional endo- and aminopeptidase (Chen et al., 2019) or by unspecific housekeeping aminopeptidases (Krawczyk et al., 2012), which sometimes act in concert with the prolyl peptidase encoded in the lanthipeptide BGC (Völler et al., 2013). Genes encoding two types of peptidases, PtrB-like (cl34357) and PqqL-like (cl33975), colocalized, respectively, with group IV *lanKC* genes and the AncKC-like clade in group II (Figure 7B), making them good candidates for leader peptide removal.

Genes encoding ABC transporters responsible for export or self-immunity are often found in lanthipeptide BGCs (Alkhatib et al., 2012). Our analysis revealed that genes coding for MdlB-like ABC transporters colocalize with ca. 70% *lanKC* loci in *Firmicutes*. A pair of genes encoding the ABC-2 type permease and the catalytic CcmA-like ATPase subunit was typical to the AncKC clade (Figure 7B and Table S4). Further studies are required for elucidating their role in export or other functions.

The andalusicin bioactivity depends on the methylation of its N-terminal residue. We analyzed the co-occurrence of genes encoding the AncMT-like family methyltransferases (cl17173) and LanKC enzymes.
Motifs found in precursor peptides associated with precursor peptide genes found in corresponding BGCs is shown in column A#. The number of predicted peptidases, transporters, and methyltransferase is indicated by closed circles. Genus names of LanKCs from the andalusicin-like clusters (Figure 7B). As a number of peptidases belonging to this family are encoded in the bioinformatics analysis revealed that genes encoding PqqL-like peptidases are frequently co-localized with another unique feature of andalusicin A is dimethylation of its N-terminal residue. Similar to the linear aza- line-containing peptide plantazolicin (Molohon et al., 2011) and the linaridin cycemicin (Claesen and Bibb, 2010), N-terminal dimethylation is required for andalusicin bioactivity. Andalusicin A without this tailoring modification showed weak or no antimicrobial activity, while the dimethylated form inhibited the growth of various Gram-positive bacteria, including S. aureus. Our preliminary data indicate that andalusicin A causes severe damage to cellular membrane of the sensitive B. cereus cells, however, the exact mechanism of its antimicrobial activity remains to be determined.

We identified the gene cluster responsible for biosynthesis of andalusicin and determined the minimal set of genes required for its production. The andalusicin BGC comprises genes coding for a LanK-like class III lanthionine synthetase AncKC, a methyltransferase AncMT, and seven genes encoding three nearly identical precursor peptides. Expression of three genes, ancKC, ancMT, and ancA1 is sufficient for andalusicin A synthesis in a heterologous host. Two genes encoding a permease (EVG22_16,520) and an ATP-binding protein (EVG22_16,515) that may form a putative two-component ABC transporter, are located upstream of ancKC. Another gene, encoding a putative MdhB-like ABC transporter (EVG22_16,530), is located downstream of the ancMT gene. Phylogenetic analysis shows that genes coding for both kinds of transporters are frequently associated with andalusicin-like BGCs (Figure 7B). Analogously to nisin BGC (Siegers and Entian, 1995), one of the transporters may be responsible for lanthipeptide secretion, while another can serve as an immunity determinant. Unfortunately, we were unable to establish whether EVG22_16,515, EVG22_16,520, and EVG22_16,530 genes are part of the anc cluster.

Similar to most other studied class III lanthipeptide BGCs, a gene required for leader peptide processing is absent from andalusicin BGC. Since heterologous production of andalusicin in B. subtilis resulted in ineffective (albeit correct) processing of the leader peptide, we speculate that the leader can be removed by a dedicated peptidase(s) encoded elsewhere in the genome of the producer strain (Chen et al., 2019). Our bioinformatics analysis revealed that genes encoding PqqL-like peptidases are frequently co-localized with andalusicin-like clusters (Figure 7B). As a number of peptidases belonging to this family are encoded in the genome of B. thuringiensis sv. andalousiensis NRRL B23139, at least one of them may be responsible for the cleavage of the leader part of andalusicin.
Phylogenetic analysis shows that methyltransferases are not common in class III lanthipeptide BGCs from Firmicutes. Indeed, the acquisition of a methyltransferase gene appeared to be a single event that happened relatively early in the evolution of andalusicin-like lanthipeptides. It is worth noting that the methyltransferase gene was lost in two daughter clades of andalusicin-like LanKCs. Further studies will show if products of clusters from these clades are functional and whether N-terminal methylation can impact on the biological activity of these compounds.

Taken together, andalusicin is the first representative of a large group of firmicute class III lanthipeptides that possess a unique structure and a secondary modification rarely found in lanthipeptides. The N-terminal methylation required for andalusicin bioactivity, if installed on other lantibiotics, may stimulate their activity and thus can find practical application.

Limitations of the study
We characterized andalusicin, the first labionin-containing class III lantibiotic from Firmicutes and identified its biosynthetic gene cluster. Andalusicin contains two methyl groups on its N terminus, a modification previously unknown for this class of lanthipeptides. Under standard laboratory cultivation conditions, the production level of andalusicin A was relatively low. This precluded further physicochemical analyses of this compound. Importantly, at this stage, we were unable to conduct crystallization experiments to establish the absolute conformation of the compound. Phylogenetic analysis of LanKC encoded in prokaryotic genomes showed that andalusicin is a representative of a large group of structurally similar lanthipeptides. Genes likely responsible for transport, leader peptide removal, and immunity were predicted, but these predictions remain to be experimentally verified. Andalusicin is bioactive against B. cereus and closely related species. Further investigations will be required to uncover the exact mechanism of andalusicin antibacterial activity and the role of N-terminal dimethylation in lanthipeptide stability, affinity to the target, or cellular permeability.

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Supplemental information can be found online at https://doi.org/10.1016/j.isci.2021.102480.

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DECLARATION OF INTERESTS
The authors declare no competing interests.

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# STAR METHODS

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Bacterial and virus strains** | | |
| Bacterial strains are listed in Table S5 | | N/A |
| **Deposited data** | | |
| Whole genome sequence of Bacillus thuringiensis sv. andalusiensis NRRL B23193 | NCBI | GenBank: NZ_CP035727.2 |
| Annotated andalusicin biosynthetic gene cluster | MIBiG | MIBiG: BGC0002111 |
| **Experimental models: organisms/strains** | | |
| Bacillus thuringiensis sv. andalusiensis NRRL B23193 | NRRL | NRRL B-23139 |
| **Oligonucleotides** | For oligonucleotides, see Table S6 | N/A |
| **Recombinant DNA** | | |
| pHT01 vector | MoBiTech, GmbH | cat# PBS001 |
| pET_His6_TEV_LIC | Scott Gradia, Addgene | RRID:Addgene_29653 |
| pHT-anckCA1 | This study | N/A |
| pHT-anckCA1(S17A) | This study | N/A |
| pHT-anckCA1(S20A) | This study | N/A |
| pHT-anckCA1(S17A:S20A) | This study | N/A |
| pHT-anckCA1MT | This study | N/A |
| pHT-anckCA1(S20A)MT | This study | N/A |
| pET_His6_ancMT | This study | N/A |
| **Software and algorithms** | | |
| ImageJ | (Schneider et al., 2012) | https://imagej.nih.gov/ij/ |
| SPAdes v. 3.9.1 | (Bankevich et al., 2012) | https://cab.spbu.ru/software/spades/ |
| RAST | (Overbeek et al., 2014) | https://rast.nmpdr.org/rast.cgi |
| MEME Suite v. 5.0.2 | (Bailey et al., 2009) | https://meme-suite.org/meme/tools/meme |
| EBI-EST | (Gerlt et al., 2015) | https://efi.igb.illinois.edu/efi-est/ |
| Cytoscape | (Shannon et al., 2003) | https://cytoscape.org/ |
| MMseqs2 version 12.113e3 | (Steinberger and Söding, 2017) | https://github.com/soedinglab/MMseqs2 |
| MUSCLE v3.8.31 | (Edgar, 2004) | http://www.drive5.com/muscle/ |
| ClipKIT v1.0.7 | (Steenwyk et al., 2020) | https://github.com/JLSteenwyk/ClipKIT |
| RaxML | (Stamatakis, 2014) | https://cme.h-its.org/exelixis/web/software/raxml/ |
| Batch CD-Search | (Lu et al., 2020) | https://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi |
| iTOL | (Letunic and Bork, 2019) | https://itol.embl.de/ |
| SciPy v. 1.3.1 | (Virtanen et al., 2020) | https://www.scipy.org/ |
| statsmodels v. 0.10.1 | (Seabold and Perktold, 2010) | https://www.statsmodels.org/stable/index.html |
| Custom scripts for bioinformatic analysis | This study | https://github.com/bikdm12/andalusicin |

## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources should be directed to the lead contact, Konstantin Severinov (severik@waksman.rutgers.edu).
Materials availability
Plasmids generated in this study will be made available by the lead contact upon reasonable request.

Data and code availability
The accession number for the assembled genome sequence of *B. thuringiensis* sv. *andalousiensis* B23139 reported in this paper is GenBank: NZ_CP035727. The accession number for the annotated andalusicin biosynthetic gene cluster is MIBiG: https://mibig.secondarymetabolites.org BGC0002111.

Table S4 includes BGCs, CDD IDs, and manually predicted precursor peptides analyzed in this study. Data used for the sequence similarity network construction was retrieved from Walker et al., 2020. Custom Python scripts used for bioinformatic and statistical analyses are available at https://github.com/bikdm12/andalusicin.

EXPERIMENTAL MODEL AND SUBJECT DETAILS
Bacterial cultures
The bacterial strains used in this study are listed in the Table S5.

*B. thuringiensis* sv. *andalousiensis* NRRL B23139 was grown aerobically in 2xYT medium (1.6% tryptone, 1% yeast extract, 0.5% NaCl, pH 7.0) at 30°C for 16 h. Aliquots (150 µl) were spread onto the 2xYT agar plates and incubated at 37°C for 3 days. Cells were collected and used for andalusicin extraction. *B. subtilis* 168 was used as a heterologous host for the production of andalusicin and its mutant forms. *B. subtilis* cells were transformed with pHT01 vector and its derivatives and grown on the 2xYT agar plates supplemented with 10 µg/mL chloramphenicol at 30°C.

METHOD DETAILS
Plasmid construction
The sequences of oligonucleotides used in this study are listed in the Table S6.

The *B. thuringiensis* sv. *andalousiensis* NRRL B23139 genomic DNA was used as a template for PCR. Phusion DNA polymerase, T4 DNA ligase, and restriction endonucleases were obtained from ThermoFisher Scientific, USA. Custom oligonucleotide synthesis was performed by Evrogen, Russia.

A sequence containing EagI-PstI restriction sites was introduced into pHT01 vector using the whole-plasmid PCR with pHT_FPst and pHT_REagPst primers, followed by digestion with PstI endonuclease and self-ligation of the resulting PCR product. For the heterologous expression of ancKC-ancA1 genes, the corresponding DNA fragment was amplified with ancKC_BamHI_F and ancA1_Eagl_R primers. For introduction of mutations in ancA1, PCR was performed with the same forward primer and one of ancA1_S17A_Eagl_R (S17A substitution), ancA1_S20A_Eagl_R (S20A substitution), or ancA1_S17A-S20A_Eagl_R (for double S17A and S20A substitution) reverse primers. The PCR products were digested with BamHI and Eagl restriction endonucleases and inserted into the pHT01-NotI vector linearized with the same endonucleases. To complement the minimal cluster with the methyltransferase gene ancMT, its coding sequence was amplified from genomic DNA with Pre_ancMT_Eagl_F and ancMT_PstI_R primers; the PCR product was digested with Eagl and PstI restriction endonucleases. The ancMT PCR was combined with the BamHI and Eagl digested ancKC-ancA1 or ancKC-ancA1_S20A PCR product, the pHT01-NotI vector linearized with BamHI and PstI, and ligated and transformed into *E. coli* DH5α cells.

For the recombinant AncMT methyltransferase production, its coding sequence was amplified from the genomic DNA with ancMT_His6_LIC_F and ancMT_His6_LIC_R primers; pET_His6_TEV_LIC vector was linearized with SspI restriction enzyme. Digested PCR product and pET_His6_TEV_LIC vector were assembled with Gibson Assembly Master Mix (NEB) to create a construction encoding N-terminal fusion protein with a hexahistidine (His6) tag. Ligation mixtures were transformed into *E. coli* DH5α cells.

Plasmids were purified using GeneJET Plasmid Miniprep Kit (ThermoFisher Scientific, USA) and transformed into appropriate bacterial strains for expression. Vectors used in the study are listed in the Table S7.
Peptide products expression and purification

*B. thuringiensis* cells (~7 g wet weight) were harvested from the agar plates, resuspended in 50 mL of 70% (v/v) methanol, and incubated at 37°C for 3 hours with occasional shaking. Cells were removed by centrifugation (3,200 x g, 4°C for 20 min), the supernatant was collected and evaporated in a centrifugal vacuum concentrator (Genevac EZ-2, SP Scientific, USA). The dried cellular extract was dissolved in 30 mL of buffer A (0.1% trifluoroacetic acid (TFA)/30% acetonitrile), briefly centrifuged to remove the insoluble material, and applied to a C18 gravity column (Mega Bound Elut-C18 10 g, Agilent Technologies, USA). The column was washed with 50 mL of buffer A, followed by elution with 25 mL of buffer B (0.1% TFA in 50% acetonitrile). Eluted fractions were dried and dissolved in 2 mL of 40% acetonitrile and subjected to further purification using HPLC.

HPLC purification of peptide compounds was performed using a Zorbax Eclipse Plus C18 column (5-μm, 4.6 x 250 mm, Agilent Technologies, USA), connected to 1220 Infinity II LC system (Agilent Technologies, USA). A linear gradient of 45 to 60% acetonitrile in 0.1% TFA was applied for 30 min. Chromatographic peaks were detected by the absorption at 280 nm. Fractions were collected and tested for biological activity in the agar diffusion test as described below. Fractions containing andalusicin or its forms were additionally purified using the same column and linear gradient of acetonitrile (40-60%) in 0.1 M triethylamine acetate buffer (pH 6.0) for 30 min. Purified compounds were dried and stored at -20°C.

AncMT activity

Recombinant AncA was produced in *E. coli* BL21(DE3) transformed with the pET_His6_TEV_LIC plasmid. The cells were grown in 200 mL of 2xYT medium supplemented with 100 μg/mL ampicillin to OD600 = 0.5. Protein expression was induced with 0.2 mM IPTG, and incubation was continued at 18°C, 180 rpm for 16 h. The cells were harvested by centrifugation at 8,000 x g, resuspended in an ice-cold wash buffer (20 mM Tris-HCl, 500 mM NaCl, 10 mM MgCl2, 5 mM imidazole, pH 8.0), supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF), and disrupted by sonication. The lysate was cleared by centrifugation at 20,000 x g at 4°C for 15 min and applied to a pre-equilibrated Talon CellThru Co2+ resin (Clontech-Takara Biotechnology, USA). The resin was washed with 10-bed volumes of the wash buffer, followed by elution with 5-bed volumes of the elution buffer (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 10 mM MgCl2, 0.5 M imidazole, 10% glycerol). Eluted protein fractions were flash-frozen in liquid nitrogen and stored at -80°C. The purity of AncMT was determined by SDS-PAGE; the identity of the protein was confirmed by tryptic peptide fingerprint using MALDI-TOF-MS.

In vitro methylation assay was conducted in a reaction buffer (50 mM Tris-HCl, pH 7.0) containing 3 mM S-adenosylmethionine (SAM), 5 μM AncMT, and 5 μM desmethyl andalusicin A at 37°C for 3 h. Prior to mass spectrometry analysis, reaction mixtures were desalted using P10 ZipTip C18 (Millipore, Ireland) according to the manufacturer’s protocol.

Andalusicin antimicrobial activity

The minimal inhibitory concentration of andalusicin A against *B. cereus* ATCC 4342 was measured in a broth dilution test. Andalusicin was serially diluted in DMSO and added to the wells containing 200 μl of LB media inoculated with 10⁶ cells of *B. cereus* ATCC 4342. The final concentration of DMSO in the wells was 3%. Plates were incubated at 37°C with moderate shaking (30 rpm) for 16 hours.

The antimicrobial activity of andalusicin on a panel of bacterial strains was determined in agar diffusion assay. Soft LB agar plates were prepared by seeding of 20 mL of molten 0.5% LB agar with 50 μL of overnight bacterial culture; 3 μl-drops of 2.5 mg/mL solutions of andalusicin A and its desmethyl form were deposited on the surface of the solidified seeded soft agar. Size of growth inhibition zone was evaluated after 24 h of
incubations at 30°C, ranged and converted into “+”, “++”, “+++”, where “+” corresponds to the smallest zone, low inhibitory activity and “+++” corresponds to the largest zone and strongest inhibitory activity. All measurements were performed in triplicates.

**Microscopy**

The overnight culture of *B. cereus* ATCC 4342 was diluted 1:50 in fresh LB medium and allowed to grow at 28°C with vigorous shaking to OD600 = 0.4. 100-μL aliquots of the cells were combined with the corresponding fluorescent dyes and 1.5 μL of either inhibitor (1.34 μg/mL nisin in 100% DMSO; 4.36 μg/mL andalusicin A in 100% DMSO; 33 mM carbonyl cyanide 3-chlorophenylhydrazone (CCCP) in 100% DMSO) or 100% DMSO as a negative control. The fluorescent dyes were used at the final concentration of 1 μg/mL for 4’,6-diamidino-2-phenylindole (DAPI) and 30 mM Propidium Iodide (PI). After 30 min of incubation at 28°C, 1-μL aliquots of the treated cells were applied to agarose pads (1.2% agarose in PBS) and imaged using Nikon Eclipse Ti microscope equipped with the Nikon Plan Apo VC 100X1.40 oil objective and Nikon DS-Qi2 digital monochrome camera. Images were processed using ImageJ software (Schneider et al., 2012).

**Chemical modifications**

Chemical modification of the Dha/Dhb residues in andalusicin with β-mercaptoethanol was performed according to the procedure described in (Meyer et al., 1994). Lanthipeptide (0.2 mg) was dissolved in the reaction mixture consisting of 280 μL ethanol, 200 μL water, 65 μL 5 M NaOH, and 60 μL β-mercaptoethanol and incubated at 50°C for 2 h. After the incubation completion, the reaction mixture was concentrated by rotary evaporation to 1/10 of the initial volume, desalted using P10 ZipTip C18 (Millipore, Ireland) according to manufacturer’s protocol, and subjected to MALDI-TOF-MS analysis.

Andalusicin treatment with Ni2B was carried out as described in (Martin et al., 2004). Andalusicin A (0.2 mg) and NiCl2 (1 mg) were dissolved in 2 mL of 50% (v/v) methanol and combined with NaBH4 (1.0 mg). The mixture was incubated at 50°C for 1 h with occasional vortexing and then centrifuged to remove the Ni2B precipitate. The supernatant was transferred into a fresh tube; the residual peptide was additionally extracted from the Ni2B precipitate by adding 50% (v/v) methanol. The supernatants were combined, dried in a centrifugal vacuum concentrator (Genevac EZ-2, SP Scientific, USA). For MALDI-TOF-MS analysis, the lanthipeptide was dissolved in a 50% (v/v) acetonitrile.

**Physicochemical methods**

UV absorbance spectrum of a 32-micromolar solution of andalusicin A in 50% acetonitrile, pH 5.0 was recorded using NanoDrop 2000 UV-Vis spectrophotometer (ThermoFisher Scientific, USA). The optical rotation value was measured on a Russian State primary standard of unit of polarization plane rotation angle polarimeter (ckp.vniiofi.ru, VNIIOFI). Measurement was performed in a 10-cm microcell at 20°C, He-Ne laser (632 nm), compound concentration 0.9 mg/mL in 50% acetonitrile.

For MALDI-TOF-MS analysis, sample aliquots were mixed (2:1) with the matrix solution (20 mg/mL 2,5-dihydroxybenzoic acid (Sigma-Aldrich), 0.5% TFA in 30% acetonitrile) on a steel target. M/z values of molecular ions were measured in reflector mode on UltraflexXtreme II MALDI-TOF-TOF (Bruker Daltonics) equipped with Nd laser. The accuracy of the obtained masses was within 0.1 Da. Spectra of fragmentation were obtained in LIFT mode; the accuracy of product ions measurement was within 1 Da range. Mass-spectra were processed using FlexAnalysis 3.2 software (Bruker Daltonics, Germany) and analyzed manually. For high-resolution mass spectrometry andalusicin A was diluted to 0.5 μg/mL in 0.1% formic acid/ 25% acetonitrile. The exact mass was measured on FTICR MS Apex Ultra (Bruker Daltonics).

The NMR sample was prepared by dissolving 3.5 mg of andalusicin in 200 μL DMSO-d6 (Sigma-Aldrich, US), and after adding 1 mM of TMS for spectral referencing, it was introduced in a 3nm NMR tube. All spectra were recorded at 30°C on an 800 MHz AvanceIII NMR spectrometer equipped with a cryogenic QCP probe head (Bruker, Billerica, USA). Homonuclear NOESY and TOCSY spectra were acquired as a matrix of 16k x 512 complex points, with a 1 s relaxation delay. The TOCSY spin lock time was set to 60 ms, and 4 scans per increment were recorded. For the NOESY spectrum, the mixing time was set to 200 ms, and 32 scans per increment were recorded. 1H-13C spectra were acquired with the standard Bruker pulse sequences (hsqcetgpsisp2.2, hsqcdietgpsisp.2, and hmbcetgpl3nd) as matrices of 4k x 512 complex points. In the
13C dimension, the carrier was set to 40 ppm and the spectral window to 80 ppm. Spectra were acquired with 32 scans for the HSQC sequences and 64 scans for the HMBC.

All spectra were transformed with the Bruker topspin3.5 program, and the assignment was manually performed with the same software.

Whole-genome DNA sequencing, genome assembly, and annotation

Genomic DNA of *B. thuringiensis* sv. *andalousiensis* NRRL B23139 was extracted with GeneJET Genomic DNA Purification Kit (ThermoFisher Scientific, USA) according to the manufacturer’s protocol. The library was prepared using NEBNext Ultra II kit (New England Biolabs, USA) and sequenced using HiSeq platform (Illumina, USA). Genome assembly was performed using SPAdes v. 3.9.1 (Bankevich et al., 2012) with *B. thuringiensis* HD521 genome sequence (GenBank: CP010106.1) (Li et al., 2015) as a reference. Genome annotation was performed using RAST software (Overbeek et al., 2014). Genes encoding andalusicin precursor peptides were identified using BLASTp against products of all ORFs with a partial sequence of the peptide predicted by MALDI-TOF-MS/MS used as a query.

Bioinformatic analysis

A dataset of LanKC sequences and corresponding automatically predicted precursor peptides was retrieved from work by (Walker et al., 2020). 1,646 class III LanKC sequences were acquired from the NCBI Protein database (https://www.ncbi.nlm.nih.gov/protein/). The sequence of andalusicin LanKC (AncKC) was added, and the resulting dataset was used for a sequence similarity network construction using the EFI-EST web tool (Gerlt et al., 2015) with a strict alignment score cutoff (130, equivalent to an e-value of 10^(-130)). For visualization of the network, the Cytoscape program was used (Shannon et al., 2003). The cluster (connected component) containing AncKC was selected for further analysis (Group II, Figure 7A). Another cluster (Group IV, Figure 7A) consisting mainly of LanKC from Firmicutes was also included in phylogenetic reconstruction to serve as an outgroup for tree rooting. Protein sequences were clustered using MMseqs2 version 12.113e3 (Steinegger and Söding, 2017) with 90% identity and 90% coverage cutoffs to remove redundancy. Obtained representative sequences were trimmed to C-terminal 400 amino acids representing LanC domains and aligned using MUSCLE v3.8.31 (Edgar, 2004) with default settings. Alignment was then manually corrected, and highly variable C- and N-terminal portions were removed. Columns containing more than 90% of gaps were removed using ClipKIT v1.0.7 (Steenwyk et al., 2020). Phylogenetic tree was reconstructed using the maximum likelihood method as implemented in RaxML (Stamatakis, 2014). Reconstruction was performed with the LG amino acid substitution model, gamma-distributed evolutionary rates, and a rapid bootstrapping that converged after 500 iterations according to autoMRE criterion. All proteins encoded within 10 kbp upstream or downstream to the *lanKC* present on the tree were obtained from RefSeq database on February 2021 (https://www.ncbi.nlm.nih.gov/refseq/) and functionally annotated using conserved domain database (CDD) database via Batch CD-Search web server (Lu et al., 2020).

Dataset for de novo motif discovery consisted of the precursor peptides associated with all LanKCs from the network clusters selected for the phylogenetic analysis. For LanKCs shown on the tree, the prediction of ORFs encoding potential precursor peptides was refined manually (Table S4). An ORF was considered as encoding a precursor peptide if it was preceded by a strong ribosome binding site, was located in the proximity to *lanKC*, and its product harbored characteristic leader motif LxLQ described previously (Müller et al., 2011). Prior to motif discovery the duplicates were removed from the dataset via clustering with MMseqs2 (Steinegger and Söding, 2017) with 95% identity and 95% coverage cutoffs. Motifs in the representative precursor peptide sequences were identified using MEME v5.0.2 (Bailey et al., 2009) with the following parameters: any number of sites per sequence, minimum motif width 6, maximum width 60, and 30 minimum sites per motif. Manually predicted sequences of precursor peptides associated with LanKCs present on the phylogenetic tree were then scanned for the presence of motifs with MAST (Bailey et al., 2009) using per sequence composition in significance calculation and omitting motifs with e-value greater than 0.05. The phylogenetic tree, along with gene co-occurrence and precursor peptide motif architectures was visualized using iTOL (Letunic and Bork, 2019). Custom python scripts used in this study are available at https://github.com/bikdm12/andalusicin.
QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis was performed in Python 3.7 using SciPy 1.3.1 (Virtanen et al., 2020) and statsmodels 0.10.1 (Seabold and Perktold, 2010) packages. The proportion of cells with the compromised membrane was calculated by manual counting the PI-positive and DAPI-positive cells. At least 3 independent measurements were performed for each of the inhibitors (Table S2). First, we compared the replicates using Fisher’s exact test. For all inhibitors, we found no difference between replicates at the significance level 0.05 (with Bonferroni multiplicity correction). Thus, we pooled the replicates and conducted another series of Fisher’s exact tests with Bonferroni adjustment and the significance level 0.05. No significant difference was observed only between negative control (DMSO) and CCCP samples. The 95% confidence intervals for the proportions of PI-stained cells were calculated using Wilson method.