Bacteriocins represent a rather underutilized class of antimicrobials despite often displaying activity against many drug-resistant pathogens. Lantibiotics are a post-translationally modified class of bacteriocins, characterized by the presence of lanthionine and methyllanthionine bridges. In this study, a novel two-peptide lantibiotic was isolated and characterized. Formicin was isolated from *Bacillus paralicheniformis* APC 1576, an antimicrobial-producing strain originally isolated from the intestine of a mackerel. Genome sequencing allowed for the detection of the formicin operon and, from this, the formicin structural genes were identified, along with those involved in lantibiotic modification, transport and immunity. The identified bacteriocin was subsequently purified from the bacterial supernatant. Despite the degree of conservation seen amongst the entire class of two-peptide lantibiotics, the formicin peptides are unique in many respects. The formicin α peptide is far less hydrophobic than any of the equivalent lantibiotics, and with a charge of plus two, it is one of the most positively charged α peptides. The β peptide is unique in that it is the only such peptide with a negative charge due to the presence of an aspartic acid residue in the C-terminus, possibly indicating a slight variation to the mode of action of the bacteriocin. Formicin also displays a broad spectrum of inhibition against Gram-positive strains, inhibiting many clinically relevant pathogens such as *Staphylococcus aureus*, *Clostridium difficile* and *Listeria monocytogenes*. The range of inhibition displayed against many important pathogens indicates a potential therapeutic use against such strains where antibiotic resistance is such a growing concern.

**INTRODUCTION**

With the increased prevalence of many drug-resistant bacterial strains, the development of new antimicrobials is becoming a growing necessity. One such class of antimicrobials that appear to be underrepresented in clinical applications are bacteriocins (Cotter *et al.*, 2013). Unlike traditional antibiotics, bacteriocins are gene-encoded, ribosomally synthesized peptides, making them suitable for genetic manipulation, with the potential for novel and specialized drug design (Gillor *et al.*, 2005). The spectrum of inhibition of bacteriocins can range from broad to narrow, the latter may allow for highly targeted antibacterial therapies that may reduce the collateral damage associated with the use of broad-spectrum antibiotics (Rea *et al.*, 2011).

The lantibiotics (*lanthionine-containing antibiotics*) comprise a well-studied class of bacteriocins, the most notable of which is nisin (Rogers, 1928), which is commonly used as a food preservative. Lantibiotics are classified based on the presence of lanthionine or methyllanthionine bridges. In these peptides, serine and threonine residues are post-translationally modified and dehydrated to form 2,3-didehydroalanine (Dha) and 2,3-didehydrobutyrine (Dhb) residues. The thiol group of a cysteine residue subsequently reacts with the Dha or Dhb residues resulting in the formation of lanthionine or methyllanthionine thioether cross-links (Xie & van der Donk, 2004).
The lantibiotic gene cluster encodes an array of genes required for modification, regulation and transport of the bacteriocin. Lantibiotics are divided into classes depending on the mechanism by which they are synthesized. Class I lantibiotics encode the enzymes LanB and LanC within the bacteriocin operon where LanB catalyses the dehydration of the serine and threonine residues, whilst LanC catalyses the cyclization of the lanthionine rings. In Class II lantibiotics, LanM alone catalyses both dehydration and cyclization of the lantibiotics (Willey & van der Donk, 2007). LanR and LanK play key roles in the regulation of lantibiotic production (Lee et al., 2011). Once the mature lantibiotic is produced, its cleavage and transport are carried out by LanP and LanT, respectively (Escano et al., 2015). In some cases, LanT can carry out both leader sequence cleavage and peptide secretion functions (Furgerson Ihnken et al., 2008). Immunity to lantibiotics can be afforded by immunity proteins such as the lipoprotein LtnI that likely binds the secreted lantibiotic and the ABC transporter LanFEG that transports bacteriocin peptides from the membrane to the extracellular medium. Here LanF binds and hydrolyses ATP that provides the energy required for the transport of the bacteriocin through the LanEG membrane complex (Stein et al., 2005; Takala et al., 2004; Alkhatib et al., 2012). For a review on this class of bacteriocins, see Willey & van der Donk (2007).

Within the lantibiotic class of bacteriocins exists a small subgroup of two-peptide lantibiotics. Such bacteriocins are produced by an array of genera, including Staphylococcus and Lactobacillus (Navaratna et al., 1998; Holot al., 2001). Interestingly, of the few two-component lantibiotics that have been described, two of these bacteriocins identified prior to this study are produced by Bacillus species. Bacillus species are known to produce a vast range of antimicrobials, whether antibiotics (e.g. gramicidin, bacitracin) or bacteriocins (e.g. thuricin CD, mersacidin) (Katz & Demain, 1977; Katz et al., 2011). Once the mature lantibiotic is produced, its cleavage and transport are carried out by LanP and LanT; respectively (Escano et al., 2015). In some cases, LanT can carry out both leader sequence cleavage and peptide secretion functions (Furgerson Ihnken et al., 2008). Immunity to lantibiotics can be afforded by immunity proteins such as the lipoprotein LtnI that likely binds the secreted lantibiotic and the ABC transporter LanFEG that transports bacteriocin peptides from the membrane to the extracellular medium. Here LanF binds and hydrolyses ATP that provides the energy required for the transport of the bacteriocin through the LanEG membrane complex (Stein et al., 2005; Takala et al., 2004; Alkhatib et al., 2012). For a review on this class of bacteriocins, see Willey & van der Donk (2007).

The mode of action of lacticin 3147 identifies a likely model for the mode of action of similarly structured lantibiotics. The α peptide of lacticin 3147 (Ltnα) resembles the globular lantibiotic mesarcidin, mirroring its activity by binding to lipid II that acts as an important docking molecule. Binding to lipid II results in a conformational change of Ltnα, which presents a site to which the β peptide (Ltnβ) can then bind. Ltnβ resembles an elongated lantibiotic, which, once recruited by Ltnα, inserts itself into the target membrane inducing pore formation resulting in cell death. Here the cooperative activity of both peptides is necessary for optimal antimicrobial activity, as the stability of the total bacteriocin–lipid II complex is important for both pore formation and the inhibition of cell wall biosynthesis (Martin et al., 2004; Wiedemann et al., 2006).

In this study, we extend the class of two-peptide lantibiotics by identifying a novel bacteriocin known as formicin that is produced by a marine isolate, Bacillus paralicheniformis APC 1576. Whilst this lantibiotic resembles the previously described two-peptide lantibiotics, it contains a number of features that differentiate it from the rest of the class.

METHODS
Isolation of bacteria from fish samples. Marine fish were caught off the coast of Ireland and stored on ice prior to analysis. The intestinal contents of the fish and a sample of the skin and gills were aseptically removed. Samples were suspended in maximum recovery diluent (Oxoid), serial dilutions were then plated on brain–heart infusion (BHI) agar (Merck) and marine media 2216 (Difco Laboratories) and were incubated aerobically at 30 °C for 3 days. Colonies were isolated from these plates and analysed for antimicrobial activity using deferred antagonism assays, whereby spots of the bacterial cultures were overlaid with 10 ml of Man, Rogosa and Sharpe agar (Difco Laboratories) seeded with 25 µl of a Lactobacillus delbrueckii subsp. bulgaricus LMG 6901 overnight culture. Colonies that displayed significant zones of inhibition were further characterized.

In this study, the strain of interest, B. paralicheniformis APC 1576, was isolated from the intestinal tract of a mackerel (Scomber sombrus) and grown on BHI aerobically at 37 °C. The strain was identified by 16S rRNA sequencing using the UniF (5′-AGAGTTTGTATCCTGGCT-CAGG-3′) and UniR (5′-AAGGCAAACCTGGTAGAT-GT-3′) primers to amplify the sequence. PCR products were cleaned using an illustra GFX PCR DNA and Gel Band Purification kit (GE Healthcare) and subsequent sequencing was completed by Cogenics (Essex).

Colonies MS. Colony MALDI-TOF MS (Axima TOF2 MALDI-TOF mass spectrometer, Shimadzu Biotech) was used to determine the molecular mass of the peptides produced as follows: cells were first grown on BHI aerobically at 37°C, the strain was then separated by centrifugation and the supernatant was subsequently concentrated. The resulting material was then reacted with a reagent containing a matrix solution, 0.5 µl of the supernatant from the cell extract was then mixed in 70 % 2-propanol/0.1 % TFA (IPA) and vortexed, the sample was then separated by centrifugation and the supernatant was subsequently concentrated. The molecular masses detected were then compared to those of known bacteriocins.

Draft genome sequencing. Genomic DNA was extracted using the GenElute bacterial genomic kit (Sigma-Aldrich) and the Nextera XT DNA kit (Illumina) was used for library preparation. The DNA was quantified using a Qubit 2.0 fluorometer. Sequencing was performed using Illumina’s MiSeq platform using paired-end 2×300 base pair reads in the Teagasc Sequencing Centre, Teagasc Food Research Centre Moorpark. Reads were assembled de novo, using SPades (version 3.1.1), resulting in 70 contigs. ORFs were identified and annotated using Prokka (version 1.1). Further manual annotation was implemented with Artemis and Artemis Comparison Tool. Genomic data are available from GenBank/EMBL under accession no. LXP000000000.

Bacteriocin identification. The bacteriocin mining tool BAGEL3 was used to identify the bacteriocin operons encoded in the genome (van
Bacteriocin purification. Cultures of *B. paralicheniformis* APC 1576 were grown statically overnight in 400 ml volumes of BHI broth aerobically at 37 °C. The cell-free supernatant (CFS) was passed through a column containing 30 g of Amberlite XAD-16 beads (Sigma-Aldrich). The column was washed with 250 ml of 35 % ethanol and antimicrobial activity eluted with 250 ml of IPA. The IPA was removed via rotary evaporation and the sample was then applied to a 10 g, 60 ml Strata C18-E solid-phase extraction (SPE) column (Phenomenex). The SPE column was washed with 90 ml of 35 % ethanol and 90 ml of IPA. The IPA was once again removed via rotary evaporation from the eluent and the sample applied to a semiprep Proteo Jupiter HPLC column (10 × 250 mm, 90 Å, 4 µm) running a 27.5–65 % acetonitrile/0.1 % TFA gradient where buffer A was 0.1 % TFA and buffer B was 90 % acetonitrile/0.1 % TFA. Fractions were collected at 1 min intervals and were subsequently analysed with MALDI-TOF MS and agar well diffusion assays as described below using *Lb. delbrueckii* subsp. *bulgaricus* LMG 6901 as the target organism to identify active fractions containing peptides of interest.

Antimicrobial assays. The antimicrobial activity of the isolated peptides was analysed using well diffusion assays against a range of indicator organisms (Table 1). Briefly, this involved seeding 20 ml of the appropriate agar with 50 µl of an overnight indicator culture; the agar was allowed to cool and 7 mm wide wells were then bored in the agar. The purified bacteriocin peptides were lyophilized and diluted separately in potassium phosphate buffer (pH 6.8) to a concentration of 50 µM. The column was washed with 90 ml of 35 % ethanol and 90 ml of IPA. The IPA was once again removed via rotary evaporation from the eluent and the sample applied to a semiprep Proteo Jupiter HPLC column (10 × 250 mm, 90 Å, 4 µm) running a 27.5–65 % acetonitrile/0.1 % TFA gradient where buffer A was 0.1 % TFA and buffer B was 90 % acetonitrile/0.1 % TFA. Fractions were collected at 1 min intervals and were subsequently analysed with MALDI-TOF MS and agar well diffusion assays as described below using *Lb. delbrueckii* subsp. *bulgaricus* LMG 6901 as the target organism to identify active fractions containing peptides of interest.

Peptide stability. The stability of the bacteriocin was determined using purified peptides. To determine the active temperature range of the lantibiotic, we treated 25 µM aliquots of the bacteriocin at 60, 70, 80, 90 and 100 °C for 30 min; a sample was also treated at 121 °C for 15 min. These samples were then tested for inhibitory activity against *Lb. delbrueckii* subsp. *bulgaricus* LMG 6901 in well diffusion assays as previously described. To determine the susceptibility of the bacteriocin to proteases, we treated 5 µM aliquots of the α and β peptides separately with proteinase K and α-chymotrypsin each at a concentration of 10 mg ml⁻¹ (Sigma-Aldrich). Samples were incubated at 37 °C for 3 h followed by treatment at 100 °C for 10 min to inactivate these proteases. Both bacteriocin peptides were then combined post-treatment to give a final total concentration of 2.5 µM; these were then screened against *Lb. delbrueckii* subsp. *bulgaricus* LMG 6901 in well diffusion assays to determine the antimicrobial activity.

RESULTS

Isolation of *B. paralicheniformis* APC 1576

*B. paralicheniformis* APC 1576 was isolated from the intestinal microbiota of a freshly caught mackerel. In an initial screen for bacteriocin producers, the strain was found to inhibit *Lb. delbrueckii* subsp. *bulgaricus* LMG 6901 in an overlay assay (Fig. 1a). In addition, CFSs also inhibited *Lb. delbrueckii* subsp. *bulgaricus* LMG 6901 in a well diffusion assay, indicating that the antimicrobial substance was secreted by the cells into the media (Fig. 1b). Colony MS was used to determine the molecular masses of the peptides produced by the cell; however, the detected peptide masses (Fig. 1c) failed to match any previously characterized bacteriocin, including lichenicidin, a bacteriocin produced by *B. licheniformis* (Begley et al., 2009). Moreover, more than one source of antimicrobial activity was found following purification of the antimicrobial peptides. MALDI-TOF MS identified a molecule with a mass of 1422.54 Da, which displayed activity against *Lb. delbrueckii* subsp. *bulgaricus* LMG 6901 once purified; this mass correlates closely with that of bacitracin, which is encoded on the genome. The production of more than one antimicrobial from *Bacillus* species is not unexpected. Therefore, in order to identify all potential antimicrobials with activity against *Lb. delbrueckii* subsp. *bulgaricus* LMG 6901, we sequenced the genome of *B. paralicheniformis* APC 1576.

Identification of a novel two-peptide lantibiotic operon

Once the draft genome was obtained, the sequence was analysed with BAGE3 and antiSMASH to identify the antimicrobials encoded. Gene clusters encoding the antibiotics bacitracin, surfactin and fengycin were found within the genome. The strain likely produces at least one of these antimicrobials, as antifungal activity was also observed against *Aspergillus niger* in overlay assays (data not shown).

A novel lantibiotic operon was also identified within the genome of the strain (Fig. 2). This operon spans approximately 17 kb and was located on a single contig of the draft bacterial genome. Two putative lantibiotic-encoding structural genes were identified on this operon. ORF1 (*frcA1*) encodes a 66-amino-acid peptide and ORF3 (*frcA2*) encodes a 71-amino-acid peptide. Analysis of the prepropeptides (including the bacteriocin leader sequence) of these lantibiotics shows that the formycin A1 prepropeptide displayed 47.8 % amino acid identity with that of the unmodified haloduracin A1 equivalent and 35.9 % identity with that of the lantibiotic mersacidin. As the putative bacteriocin appears to be a two-peptide bacteriocin, two lantibiotic modification enzymes should be present. The order of the genes in the operon would suggest that ORF2 (*frcM1*) is the modification enzyme associated with *frcA1*. Upon analysis, this ORF displayed 38.7 % identity with that of the haloduracin HalM1 modification enzyme. The second lantibiotic gene, ORF3 (*frcA2*), appears to resemble the elongated β peptides of the other two-peptide lantibiotics that are involved in membrane insertion (Wiedemann et al., 2006). Upon analysis, formycin A2 revealed 42.4 % identity with the unmodified lichenicidin LchA2 prepropeptide. ORF4 (*frcM2*) encodes the modification enzyme, which follows this
structural peptide, and displayed 33.6% identity with that of the lichenicidin LchM2 modification enzyme.

ORF5 located downstream of LchM2 is predicted to encode a lantibiotic transporter, displaying 52.5% identity with that of the haloduracin transporter, HalT. In addition to its function in bacteriocin transport, a sequence encoding a C39 peptidase domain (cd02425) can also be found within the gene, this is likely involved in the cleavage of the leader sequence from the prebacteriocin.

BLAST analysis of ORF6 identified the gene as encoding a hypothetical protein; the sequence, however, did show 28.4% identity with that of LanY encoded within the lichenicidin operon (Begley et al., 2009). ORF7, ORF8 and ORF9 all encode ABC transporter-related peptides, as do ORF11, ORF12 and ORF13. These are likely to be involved in bacteriocin immunity. ORF7 and ORF11 both encode domains resembling that of the ABC-binding cassette domain of the bacitracin resistance transporter (cd03268) and displayed 44.5% identity with that of each other. Instead of the common Q-loop motif found in the nucleotide-binding domains of such transporters, both these proteins instead encode an E-loop motif that is indicative of a different function.

Table 1. Growth conditions of indicator strains and inhibition spectrum of formicin pure peptides following well diffusion assays

MRS, de Man, Rogosa and Sharpe; BHI, brain–heart infusion; RCM, reinforced clostridial media. –, No activity; +, 0.5–1.5 mm inhibition zone; ++, 2–3.5 mm inhibition zone; ++++, ≥4 mm inhibition zone.

<table>
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<th>Species</th>
<th>Strain</th>
<th>Temp. (°C)</th>
<th>Atmosphere</th>
<th>Growth media</th>
<th>Inhibition</th>
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of lantibiotic immunity proteins (Okuda et al., 2010; Alkhatib et al., 2012). Each of the other components encodes ABC-2-type transporter domains (cl21474). The presence of these gene clusters may suggest a dual mechanism of bacteriocin immunity. Immunity to the lichenicidin bacteriocin is thought to follow a similar mechanism, with two transporters being encoded, with one showing homology to the bacitracin transporter (Dischinger et al., 2009). Such mechanisms, however, do not confer a general immunity against all two-peptide lantibiotics, as both the producers of lichenicidin (B. licheniformis ATCC 14580) and lacticin 3147 (Lc. lactis subsp. lactis DPC 3147) displayed sensitivity to formicin (Table 1).

ORF10 (frcR) that splits the transporter clusters encodes a LanR-equivalent transcriptional regulator. This gene encodes helix–turn–helix XRE family domains, crucial for binding DNA and regulating gene expression. This LanR-type protein displayed 49.4 % and 60.3 % identity with those of the regulators found within the lichenicidin and haloduracin operons, respectively. ORF14 (frcP) encodes a lanthionine-specific protease displaying 29.8 % identity with that of LicP found in the lichenicidin operon. As in lichenicidin, the LanT-like ORF (frcT) likely cleaves the N-terminal glycine leader sequence from both propeptides upon transport, whilst the LanP-like protease (frcP) possibly cleaves the six newly exposed N-terminal amino acids from the β peptide to generate the mature bacteriocin (Tang et al., 2015). The final ORF found in the gene cluster encodes a DNA damage-inducible protein.

Fig. 1. Formicin identification and activity. (a) Deferred antagonism assay against Lb. delbrueckii subsp. bulgaricus LMG 6901 identified B. licheniformis APC 1576 as an antimicrobial producer. (b) Antibacterial activity of the B. licheniformis APC 1576 CFS against Lb. delbrueckii subsp. bulgaricus LMG 6901 in a well diffusion assay. (c) Colony MALDI-TOF MS displaying the masses of the peptides produced by B. licheniformis APC 1576, allowing identification of the antimicrobials produced (3255.92 Da=Frcα (formicin); Frcβ is not seen using colony MALDI-TOF MS; 1423.94 Da=bacitracin).
Bacteriocin structure prediction and analysis

The spectrum of activity and characteristics of the bacteriocin could not be determined from the crude bacteriocin supernatant alone due to the interference from the other antimicrobials produced by the strain. Thus, it was necessary to purify the bacteriocin from the CFS in order to determine the activity of formicin. With the use of the predicted masses of the lantibiotic structural peptides identified from genomic data, it was possible to determine if the formicin peptides were present in active HPLC-derived fractions using MALDI-TOF MS.

From the purified peptides, masses of 3254.34 and 2472.06 Da were detected for the $\alpha$ and $\beta$ peptides, respectively. The predicted mass of the Frc$\alpha$ peptide based on the amino acid sequence from the genome is 3310.80 Da; the difference between the predicted and observed masses correlates with the loss of three water residues, which is most likely associated with the formation of lanthionine and methyllanthionine bridges, as well as also the possible formation of one disulfide bond, resulting in a predicted mass of 3254.80 Da.

The second mass determined by MALDI-TOF MS relates to the $\beta$ peptide of the bacteriocin. Due to the presence of the extra LanP serine protease encoded in the bacteriocin operon and the similarity formicin displays to haloduracin and lichenicidin, it is likely that the first six amino acids following the lantibiotic leader sequence are also cleaved from the formicin peptide. Once these amino acids are discounted, the predicted mass of the peptide is 2614.95 Da, a difference of 142.89 Da from the mass detected by MALDI-TOF MS. This mass difference corresponds closely with the loss of 144 Da, which would be associated with eight dehydration reactions. Using the $\beta$ peptides of lichenicidin and lacticin 3147 as templates, we predicted that the peptide is most likely to form bridges between Thr-1 and Cys-8, Thr-13 and Cys-17, Ser-19 and Cys-22 and Thr-23 and Cys-26. This would result in Thr-2, Ser-4, Ser-5 and Thr-10 being dehydrated to their respective Dha and Dhb residues, whilst Ser-24 remains unaltered (Fig. 3).

The purified peptides were screened against a range of indicator organisms to determine the spectrum of inhibition (Table 1). Purified formicin inhibited 29 of the 35 indicator strains screened, exhibiting a broad spectrum of activity against a range of bacterial genera including lactobacilli and enterococci, as well as notable pathogens such as Staph. aureus, Strep. mutans, Ls. monocytogenes, C. difficile and B. subtilis. The Frca peptide alone at a concentration of 50 µM also displayed antimicrobial activity against a number of indicators, whilst Frc$\beta$ alone displayed no detectable antimicrobial activity.
In terms of thermostability, the bacteriocin retained a high degree of activity after treatment at 100 °C for 30 min, displaying a reduction in the size of the zone of inhibition of approximately 28%. Activity was, however, lost after treatment for 15 min at 121 °C. The bacteriocin was also found to be susceptible to digestion by a-chymotrypsin and proteinase K, indicating its proteinaceous nature.

**Homology between bacteriocins**

The previously described two-peptide lantibiotics all display a degree of homology with certain conserved residues found throughout. As a result, sequence comparisons of these structural peptides were carried out with formicin to determine if this conservation extended to the new bacteriocin (Fig. 4). The results indicate that formicin complies with the conservation that is seen amongst the other bacteriocins. The mersacidin-like a peptides display the greatest levels of conservation, and this reflects the shared mode of action in specifically binding to lipid II. This homology, especially in the lanthionine and methylanthionine bridge-forming regions, confers a structural similarity in each of the peptides. The broader role of the b peptides in membrane insertion is reflected in a greater degree of divergence in the composition of these peptides. The regions of conservation that are seen amongst the b peptides extend to Frc also, with the C-terminus of the peptides showing a relatively conserved pattern of lanthionine and methylanthionine bridge formation. The N-terminus of the b peptides displays a much lower degree of conservation amongst the bacteriocins; despite this, these N-terminal regions are rich in hydrophobic amino acids, which likely play an important role in membrane insertion and pore formation.

**DISCUSSION**

Formicin represents a novel member of the class of two-peptide lantibiotics. This class of bacteriocins are themselves unusual given that the lipid II-binding and pore-forming
activities of the bacteriocin are performed by two separate peptides, whilst certain lantibiotics such as nisin and subtilin have the ability to carry out both functions on a single peptide. It is unclear as to whether these two-component lantibiotics have evolved due to a divergence of a nisin-like lantibiotic into two separate genes due to a duplication event or whether they have come about due to the convergence of a meracin-like lipid II-binding lantibiotic and a pore-forming lantibiotic. If the latter is the case, it is interesting as to how such different peptides would have evolved to depend on each other for antibacterial activity, and in some cases, lose the activity each would have shown on its own.

Sequencing of *B. paralicheniformis* APC 1576 allowed for the elucidation of the formicin bacteriocin operon (Fig. 2). Analysis of the bacteriocin operon identified two lantibiotic structural genes (*frcA1* and *frcA2*) and two modification enzymes (*frcM1* and *frcM2*) that convert the formicin structural peptides into the mature lantibiotics. Transport and leader cleavage are likely to be carried out by *frcT*, whilst *frcP* may act as a further protease, cleaving six N-terminal amino acids from Frcβ. ORF7, ORF8 and ORF9 and ORF11, ORF12 and ORF13 all predict to encode ABC transporters that are likely to comprise the strain’s immunity mechanism, protecting itself from attack by its own bacteriocin. Comparative analysis of the bacteriocin structural genes allows for the homology between bacteriocins to be determined (Fig. 4). In the case of both Frcα and Frcβ, the closest homologues are the haloduracin α and β mature peptides, displaying 71 and 39% identity, respectively. Such homology reflects the close relationship of the two producers, both belonging to the *Bacillus* genus. The differences between the formicin and lichenicidin peptides are surprisingly large, given that both are produced from related species, with the α peptides displaying 46% identity and the β peptides displaying 36% identity. This would suggest that both strains may have acquired these operons independently. The layout of the formicin operon itself differs from that of the previously characterized two-peptide lantibiotics, and transcription of the formicin operon would appear to be unidirectional whereby the genes for the structural peptides are separated by those encoding the LanM modification enzymes, an arrangement that seems to be unique to formicin. Both the haloduracin and lichenicidin structural genes (Fig. 2) would likely be transcribed in opposite directions, possibly indicating that gene inversion may have taken place. Such differences again display the evolutionary divergence seen between this class of bacteriocins.

Analysis of the primary structure of these peptides indicates that some key differences exist between the formicin peptides and other members of the class despite such strong regions of homology found throughout. The α peptide of formicin, for example, contains only five hydrophobic amino acids, whilst others in the class contain an average of nine. Whilst hydrophobic residues are crucial for membrane activity in certain bacteriocins, it has been suggested that it is the charged residues of these lantibiotics that control binding to lipid II as opposed to hydrophobic interactions. This indicates that binding of formicin to lipid II is not compromised despite its lower hydrophobicity, a fact that is supported by the activity of the α peptide independent of the β peptide (Hsu et al., 2003; Finland et al., 2006). As with the α peptides from enterocin W and plan taricin W, the α peptide of formicin contains six charged amino acids, with an overall positive charge of plus two, rendering them amongst the most highly charged in the class. Not only do these charged residues affect the structure of the peptide but also the higher positive charge may lead to an increased affinity for the anionic bacterial membrane. The formicin β peptide differs most when compared to other lantibiotic β peptides with regard to charge. As is common in this class, the N-terminal tails of the β peptides are composed largely of hydrophobic residues, crucial for membrane insertion and pore formation. Whilst the previously described β peptides all contain a positively charged C-terminus, containing Lys and Arg residues, formicin is unique in that it encodes a negatively charged β peptide. The lone charged residue found in the peptide is the
penultimate C-terminal Asp residue. This portion of the peptide is believed to be involved in the interaction between the α and β peptides (Wiedemann et al., 2006); thus, this negative residue may suggest an increased affinity for the positively charged α peptide, possibly representing a stronger complex compared to previously described pairs.

The tertiary structure of these peptides has an important functional role in the antimicrobial activity of these lantibiotics. Analysis of the N-terminal of Frcα suggests the formation of a disulfide bridge between Cys-1 and Cys-8. Whilst this has been shown to be inessential for antimicrobial activity, it may reduce the degradation of the peptide once secreted (Cooper et al., 2008). Of the lantibiotic rings believed to be formed in Frcα, only the C ring is thought to be essential, with alterations abolishing all activity completely in both haloduracin and lacticin 3147 (Cooper et al., 2008; Cotter et al., 2006). The B ring found in these α peptides has been shown to be unnecessary, which is unusual given the high degree of conservation amongst such bacteriocins, including mersacidin. Disruption of the A ring in haloduracin has been shown to reduce but not eliminate activity, thus showing that this region is important but not essential for the antibacterial activity of the bacteriocin (Cooper et al., 2008). As per analysis of the haloduracin β peptide, the A ring of the peptide has been found to be dispensable, whilst loss of the C and D rings led to a reduction in activity but not total elimination. Disruption of the B ring could not be achieved without disruption of the other ring structures (Cooper et al., 2008).

CONCLUSION

In this study, formicin, a novel member of the class of two-peptide lantibiotics has been identified. Key regions of homology, primarily those involved in lanthionine and methylthionine bridge formation, seen throughout this class have been shown to be extended to formicin. Such homology is expected to confer a similar mode of action to all lantibiotics in this class, with the α peptide of the bacteriocin binding to lipid II and subsequently recruiting the β peptide for membrane insertion and pore formation. Whilst formycin like conforms to such mechanisms, there are certain key variations differentiating it from the rest of the class. The reduction of hydrophobicity of Frcα and the unusual negative charge of Frcβ make formycin a unique member of the two-peptide lantibiotics. Further studies are required to determine the effects of such changes on the activity of the bacteriocins, as it is recognized that charge and hydrophobicity play a central role in the activity of these lantibiotics and in bacteriocins in general. Formycin itself displays a broad range of inhibition, inhibiting several clinically relevant Gram-positive pathogens, such as C. difficile, Staph. aureus, Strep. mutans and Ls. monocytogenes. With the continued progression of antibiotic resistance in pathogenic bacteria, the discovery of novel therapies against such agents is a priority and since the bacteriocin is produced by a species long associated with biotechnology applications, a straightforward route towards large-scale processing of the readily purified peptides is anticipated. Thus, formicin represents a potential novel antimicrobial therapy against a range of pathogenic bacteria.

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