

# Plantaricin W from *Lactobacillus plantarum* belongs to a new family of two-peptide lantibiotics

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**Plantaricin W (Plw) is a new two-peptide bacteriocin, from *Lactobacillus plantarum*, which inhibits a large number of Gram-positive bacteria. The two peptides, Plw $\alpha$  (comprising 29 residues) and Plw $\beta$  (comprising 32 residues), were isolated from the culture supernatants and characterized. The individual peptides had low antimicrobial activity but acted synergistically, and synergism was seen at all mixing ratios tested. The data indicate that the two peptides work in a 1:1 ratio. Chemical analyses showed that both peptides are lantibiotics, but two unmodified cysteines and one serine residue were present in Plw $\alpha$ , and Plw $\beta$  contained one cysteine residue. The Plw structural genes were sequenced and shown to encode prepeptides with sequence similarities to two other two-peptide lantibiotics, namely staphylococcin C55 and lacticin 3147. The conserved residues are mainly serines, threonines and cysteines that can be involved in intramolecular thioether bond formation in the C-terminal parts of the molecules. This indicates that these bacteriocins are members of a new family of lantibiotics with common bridging patterns, and that the ring structures play an important functional role. Based on the data a structural model is presented in which each peptide has a central lanthionine and two overlapping thioether bridges close to their C-termini.**

Keywords: two-peptide bacteriocin, lactic acid bacteria

## INTRODUCTION

Many bacteria produce bacteriocins, antimicrobial proteins or peptides that kill other strains. Several of the bacteriocins from Gram-positive bacteria are very potent, have wide inhibitory spectra and may find use as antimicrobial agents in various practical applications. In particular, the bacteriocins from lactic acid bacteria have attracted great interest because of their potential use as non-toxic and safe additives for food and feed preservation.

The bacteriocins from lactic acid bacteria are mostly small, heat-stable, hydrophobic and cationic peptides (Jack *et al.*, 1995). The peptide bacteriocins are either

**Abbreviations:** BU, bacteriocin unit; Dha, 2,3-didehydroalanine; Dhb, 2,3-didehydrobutyrine; Lan, lanthionine; MeLan, 3-methylanthionine; Plw, plantaricin W; TFA, trifluoroacetic acid.

The GenBank accession number for the sequence reported in this paper is AY007251.

normal, unmodified peptides or lantibiotics, the latter being defined as ribosomally synthesized peptides containing the thioether amino acids lanthionine (Lan) and 3-methylanthionine (MeLan). Jung (1991) divided the lantibiotics into two types: type A lantibiotics are elongated peptides with molecular masses of 2164–3488 Da, whereas type B lantibiotics are globular molecules with masses of 1959–2041. In addition to Lan and MeLan, the  $\alpha,\beta$ -unsaturated amino acids 2,3-didehydroalanine (Dha) and 2,3-didehydrobutyrine (Dhb) are frequently found in lantibiotics. The unusual amino acids in lantibiotics are formed as a result of post-translational modifications of precursor peptides. Serine and threonine residues are specifically dehydrated to give Dha and Dhb, respectively. The addition of cysteine thiol groups to the dehydrated residues leads to the formation of the intramolecular thioether bridges.

In general, the lantibiotics have wider inhibitory spectra than the non-lantibiotics. Nisin, produced by strains of *Lactococcus lactis*, is the best-studied lantibiotic and is

also the only lantibiotic in practical use as an antimicrobial agent (Gross & Morell, 1971). The search for other bacteriocins of food-grade micro-organisms (lactic acid bacteria) is ongoing. Recently, a new lantibiotic – lactacin 3147 – produced by other strains of *Lc. lactis* was reported to have potential uses in food preservation as well as in veterinary medicine (Ryan *et al.*, 1996, 1998, 1999).

Insights into the mode of action and the structure–function relationships is of great value in exploiting the antimicrobial properties of these peptides. The intramolecular rings formed by the thioether bonds give the lantibiotics unique structural properties. These bridges are probably also of great importance for their antimicrobial activities, and genetic engineering experiments have shown that mutations affecting the bridging pattern are detrimental to activity (Kuipers *et al.*, 1996). In this report, we describe the two-peptide lantibiotic plantaricin W (Plw), which possesses several properties previously unseen among bacteriocins. Our data indicate that Plw belongs to a new family of two-peptide lantibiotics with conserved bridging patterns.

## METHODS

**Bacterial strains.** The bacteriocin-producing strain *Lactobacillus plantarum* LMG 2379, originally isolated from fermenting Pinot Noir wine in Oregon, and the indicator strain *Lactobacillus sakei* NCDO 2714 were grown in MRS broth (Oxoid) at 30 °C.

**Determination of bacteriocin activity.** Sterile, cell-free culture supernatants were obtained by centrifugation (15000 g, 10 min) followed by incubation at 100 °C for 10 min. Bacteriocin activity was quantified by the microtitre plate assay (Holo *et al.*, 1991). Each well of the microtitre plate contained 200 µl MRS broth, bacteriocin fractions at twofold dilutions, and the indicator organism, *Lb. sakei* NCDO 2714 (about 10<sup>6</sup> c.f.u. ml<sup>-1</sup>). The microtitre plate cultures were incubated overnight at 30 °C, after which growth inhibition of the indicator organism was measured spectrophotometrically at 600 nm. By definition, the activity of one bacteriocin unit (BU) causes 50% inhibition (50% of the growth of the control culture lacking bacteriocin addition) in this assay. An agar diffusion assay (Cintas *et al.*, 1995) was used for the examination of the inhibitory spectrum of the bacteriocin.

**Isolation of plantaricin W.** The bacteriocin was isolated from 1 litre cultures of strain LMG 2379 grown to early stationary phase. The cells were removed by centrifugation (10000 g for 10 min). Ten millilitres of SP Sepharose (Amersham Pharmacia Biotech) was added to the supernatant and the mixture was left overnight at 4 °C with constant stirring with a magnetic stirring bar. The mixture was then transferred to a chromatographic column, the matrix was washed with 200 ml 10 mM sodium phosphate (pH 7) and the bacteriocin was eluted in 20 ml 1 M NaCl. The eluate was applied to a column containing 2 g Amberlite XAD-16 (Supelco) equilibrated with water. After washing with 10 ml water and 10 ml 40% ethanol in water, the bacteriocin was eluted in 10 ml 70% 2-propanol containing 0.1% trifluoroacetic acid (TFA). This eluate was then subjected to reverse-phase chromatography on a Resource-RPC column, using an Äkta Purifier System (Amersham Pharmacia Biotech). The peptides were eluted from the column in a water/2-propanol gradient containing

**Table 1.** Oligonucleotide primers used in the PCR and DNA sequencing

Primer	Sequence
W1	AARNNNAARTGGTGAA
W2	CCRTTRTTNCCNARRTC
W3	TCACACGAAATATTCCA
W4	GGCAAGCGTAAGAAATAAATGAG
T7	GTAATACGACTCACTATAGGG
SK2	CCGCTCTAGAACTAGTGGATC

0.1% TFA. Fractions showing synergistic activities were collected and purified separately by repeating the reverse-phase chromatography step.

**Amino acid sequencing and composition analysis.** The purified peptides were hydrolysed and analysed on an amino acid analyser as described previously (Fykse *et al.*, 1988). The amino acid sequence was determined by Edman degradation using an Applied Biosystems 477A automatic sequence analyser with an on-line 120A phenylthiohydantoin amino acid analyser (Cornwell *et al.*, 1988). To enable Edman degradation of Plw $\beta$ , the peptide was chemically modified according to Meyer *et al.* (1994).

**Mass spectral analysis.** MS analysis was performed on a Sciex API I electrospray mass spectrometer.

**PCR and DNA sequencing.** DNA was prepared by the method of Anderson & McKay (1983). Restriction enzymes and other DNA-modifying enzymes were used as recommended by the manufacturer (Promega). PCRs were performed with Dynazyme (Finnzymes) in a DNA thermal cycler (Perkin-Elmer). The DNA primers used for PCR and DNA sequencing are shown in Table 1. The PCR products were purified by agarose gel electrophoresis and extracted from the gel with the GeneClean II kit (Bio 101). The PCR products were sequenced with the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer) and an ABI PRISM 377 DNA sequencer (Perkin-Elmer). The degenerate oligonucleotide primers W1 and W2 were constructed on the basis of the amino acid sequence of Plw $\alpha$  and used in a PCR to generate a 44 bp DNA fragment. This fragment was sequenced and new primers based on its sequence were synthesized. Further sequences were obtained largely as described previously (Casaus *et al.*, 1997); DNA of strain LMG 2379 was cut with *EcoRV*, *DraI* and *RsaI*, then ligated to plasmid vector Bluescript SK II (Stratagene) cut with *HincII*. The ligation products served as templates in PCRs using *plw*-specific primers in combination with vector-specific primers; the products obtained were sequenced.

## RESULTS

### Purification and N-terminal sequence of the Plw peptides

Culture supernatants of *Lb. plantarum* LMG 2379 were inhibitory against a number of Gram-positive bacteria in the agar diffusion assay (Table 2), but Gram-negative bacteria were not inhibited (results not shown). The bacteriocin, Plw, was purified from culture supernatants as described in Methods. Little or no activity was found

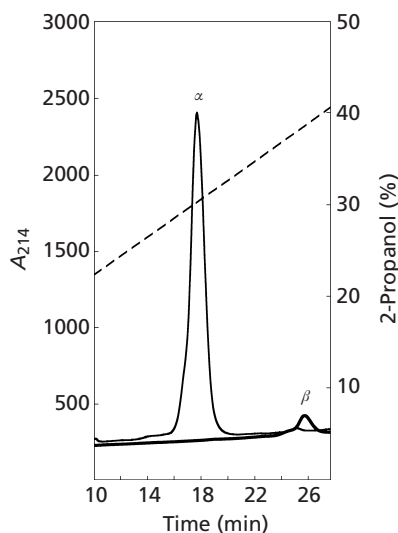
**Table 2.** Inhibitory spectrum of Plw

Inhibition was assayed in the agar-well diffusion assay, using culture supernatants of *Lb. plantarum* LMG 2379.

Species	No. of strains tested	No. of sensitive strains
<i>Lactobacillus</i> spp.	26*	22†
<i>Lactobacillus lactis</i>	6	5
<i>Oenococcus oenos</i>	4	4
<i>Leuconostoc mesenteroides</i>	7	7
<i>Pediococcus acidilactici</i>	2	2
<i>Pediococcus pentosaceus</i>	4	4
<i>Enterococcus faecalis</i>	5	4
<i>Bacillus coagulans</i>	1	0
<i>Bacillus subtilis</i>	3	0
<i>Listeria innocua</i>	1	1
<i>Listeria monocytogenes</i>	3	1
<i>Propionibacterium freudenreichii</i>	1	1
<i>Staphylococcus aureus</i>	2	2

\* 26 strains, 13 species.

† 22 strains, 13 species.



**Fig. 1.** Reverse-phase chromatograms of purified Plw $\alpha$  and Plw $\beta$ . The purified peptides were applied to a Resource-RPC column and eluted using a linear gradient of 2-propanol containing 0.1% TFA at a flow rate of 1 ml min<sup>-1</sup>.

in the individual fractions from the first step of reverse-phase chromatography, but when the fractions were mixed, high activity was recovered. It became clear that the inhibitory activity was dependent on the presence of two components. These components were easily separated by reverse-phase chromatography (Fig. 1) and were investigated by using N-terminal amino acid sequencing and amino acid composition analysis. Table 3 shows the amino acid composition of the two peptides,

Plw $\alpha$  and Plw $\beta$ . The following N-terminal sequence was obtained for Plw $\alpha$ : KKKWWNIXXDLGNNGXVXX-LXXEXQ (residues that could not be identified are shown as X).

Plantaricin W $\beta$  was blocked for N-terminal amino acid sequencing. However, after the peptide had been subjected to a series of modification reactions designed to overcome lantibiotic sequence blocks (Meyer *et al.*, 1994) a partial amino acid sequence could be obtained by Edman degradation. The following sequence was obtained of the modified peptide: XGIPX2IGAAVA-A1IAVXP22KX1K where 1 is S-propylcysteine, derived from serine or cysteine, and 2 is methyl-S-propylcysteine, derived from threonine.

### Sequences of the bacteriocin genes and elucidation of primary structure

The sequences of the genes encoding Plw $\alpha$  and Plw $\beta$  were obtained after the use of PCR and degenerate oligonucleotide primers synthesized on the basis of the amino acid sequence of Plw $\alpha$ . The sequence of the region (Fig. 2) revealed that the two genes encoding each of the peptides are located next to each other. A putative promoter was identified upstream of *plw $\beta$ A*, which is immediately preceded by *plw $\alpha$ A*, indicating that the two genes are organized in a transcriptional unit, like all other two-peptide bacteriocins studied.

As shown in Fig. 2, both genes code for probacteriocins with N-terminal extensions. The *plw $\alpha$ A* gene encodes a peptide of 59 residues, and the 30 aa N-terminal extension was identified as a leader peptide of the double-glycine type (Håvarstein *et al.*, 1994). The probacteriocin is a 29 aa peptide with a calculated

**Table 3.** Amino acid composition of the Plw peptides

Amino acid	Calculated from transcript of <i>plw<math>\alpha</math>A</i>	Found in Plw $\alpha$	Calculated from transcript of <i>plw<math>\beta</math>A</i>	Found in Plw $\beta$
Ala	0	0	5	5
Arg	0	0	2	2
Asx	5	5	0	0
Cys	5	2	4	1
Glx	2	2	0	0
Gly	2	2	3	3
His	2	2	0	0
Ile	1	1	3	3
Leu	2	2	0	0
Lys	2	2	5	5
Pro	0	0	2	2
Ser	3	1	3	0
Thr	1	0	3	0
Trp	2	–	0	–
Val	2	2	2	2
Lan + MeLan		Present (3)		Present (3)

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60
AAATGTTTTTAATAAAATATATAAAATATTGTTGATTTAAAAATGACTGTATTTAATA
120
AAAGTTGTTTCAGGTAAGCGCTTGCCAATGAACAAATATGAAAAGGGGCTTTATATATGAC
RBS M T
plw $\beta$ A
180
TAAACTAGTCGTCGTAAGAATGCTATTGCTAATTTTGTAGAACAGTCGACGAAAAAAG
K T S R R K N A I A N Y L E P V D E K S
240
TATTAATGAATCTTTTGGGGCTGGGGATCCGGAAGCAAGATCCGGAATTCATGTACAAT
I N E S F G A G D P E A R S G I P C T I
300
CGGCGCAGTGTCGCGCATCAATTGCGAGTTTGTCCAACACTAAGTGTAGTAAACGTTG
G A A V A A S I A V C P T T K C S K R C
360
TGGCAAGCGTAAGAAATAAATGAGTATCAACTTTTAGGAGGGTTTTTATGAAAATTTCTA
G K R K K - RBS M K I S
plw $\beta$ A
420
AGATTGAAGCTCAGGCTCGTAAAGATTTTTTTAAAAAAATCGATACTAAGTTCGAACCTTAT
K I E A Q A R K D F F K K I D T N S N L
480
TAAATGTAATGGTGCACAAATGCAAGTGGTGAATATTTTCTGTGATTAGGAAATAATG
L N V N G A K C K W W N I S C D L G N N
540
GCCATGTTGTACCTGTGCACATGAATGCCAAGTATCTTGTAACTAATTTGGTATAAAAC
G H V C T L S H E C Q V S C N -
600
TAAAAATAATGGGACAAAGATCAATTTAGTTAATAAAATAGTTGCATAAGCTGATATATC

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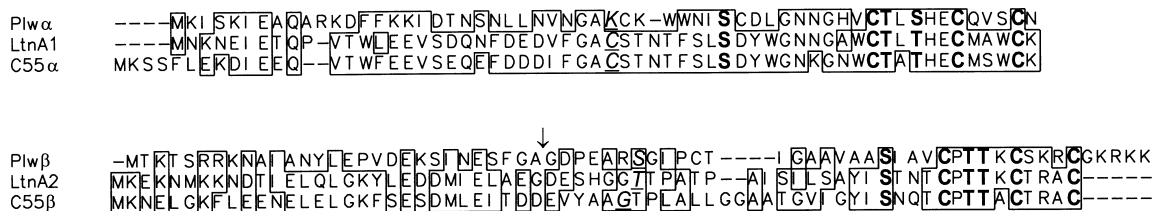
**Fig. 2.** Nucleotide sequence of the region containing the Plw structural genes. A putative promoter region is indicated in bold. Potential –35 and –10 promoter regions and ribosome-binding sites (RBS) are underlined. The translations of the two genes are also shown; the sequences corresponding to the mature peptides are in bold.

molecular mass of 3279.67 Da and a pI of 6.88. With the exception of the Cys, Ser and Thr residues, its amino acid composition corresponds well with that obtained for isolated Plw $\alpha$ . As shown in Table 3, these three residues were present in lower amounts than predicted.

The presence of Lan and MeLan in the hydrolysate identified the peptide as a lantibiotic. In lantibiotics, most Cys, Ser and Thr residues are modified. Ser and Thr residues are dehydrated and may react with Cys residues to form Lan or MeLan. The data presented in Table 3 suggest that three of the Cys residues are modified this way, and that Plw $\alpha$  contains two Lan residues and one MeLan. This would give a peptide with a calculated molecular mass of 3225 Da. The molecular mass of Plw $\alpha$  was determined, by MS, as 3223. This value is in excellent agreement with that of a peptide containing two Lan residues and one MeLan residue, especially if it is also assumed that a disulfide bridge links the remaining two Cys residues. Since they are usually modified in lantibiotics, it is noteworthy that Plw $\alpha$  contained one unmodified Ser residue as well as two Cys residues.

The role of the cystine bridge in Plw $\alpha$  was studied by performing the microtitre assay in the presence of 10 mM mercaptoethanol to reduce the disulfide bond (data not shown). The activity of isolated Plw $\alpha$  was increased about fourfold by introducing mercaptoethanol into the assay. However, the thiol reagent caused no activity increase with Plw $\beta$  or with mixtures of Plw $\alpha$  and Plw $\beta$ .

The data obtained by amino acid sequencing of the modified Plw $\beta$  were combined with amino acid composition analysis and DNA sequencing to elucidate the primary structure of the peptide. From Fig. 2, it can be seen that the 32 aa C-terminal part of the Plw $\beta$  translation product corresponds to the isolated peptide. This peptide has a calculated molecular mass of 3207 Da and a pI of 9.96. With the exception of the three amino acids that are usually modified in lantibiotics, there is a good



**Fig. 3.** Alignment of the Plw prepeptides with the corresponding peptides of lacticin 3147 and staphylococcin C55. Identical and similar residues are boxed. Residues corresponding to the N-terminal residues of the mature peptides are underlined and shown in italics. The arrow points to the putative primary processing site for Plw $\beta$ . Residues suggested as being involved in thioether ring formation are shown in bold.

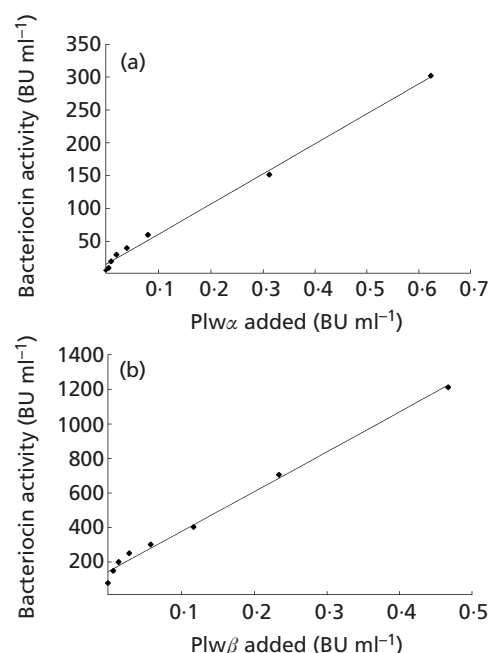
correlation between the amino acid composition of the unmodified peptide and that of Plw $\beta$  (Table 3). For Plw $\alpha$ , the data indicate that three Cys residues are involved in Lan/MeLan formation. Furthermore, all six hydroxy acid residues appeared to be modified, probably dehydrated. The molecular mass of Plw $\beta$  was found, by MS, to be 3099 Da, which is in agreement with that for a modified peptide containing six dehydrated residues.

### Homology with other bacteriocins

Computer-aided homology searches revealed that the Plw $\beta$  prepeptides have sequence similarities with the prepeptides of two other two-peptide bacteriocins, namely lacticin 3147 (produced by *Lc. lactis*) and staphylococcin C55 (produced by *Staphylococcus aureus*). Both of these bacteriocins are two-peptide lantibiotics (Dougherty *et al.*, 1998; Navaratna *et al.*, 1998, 1999; Ryan *et al.*, 1999). In Fig. 3, the corresponding prepeptides have been aligned. Staphylococcin C55 and lacticin 3147 are much more closely related to each other than to Plw, having 86.7 and 43.0% sequence identity for the  $\alpha$  and  $\beta$  propeptides, respectively. The Plw $\alpha$  propeptide showed 31.2% and 40.0% identity to the corresponding peptides of staphylococcin C55 and lacticin 3147, respectively. The values for the Plw $\beta$  propeptide and its respective homologues were 31.6 and 26.5%. The alignments also show that most of the residues conserved in all three bacteriocins are located in the C-terminal parts of the peptides. Furthermore, they are amino acids that contribute to structural rigidity: Cys, Ser and Thr residues that can be involved in bridge formation and, in the case of the  $\beta$  peptides, also Pro residues.

### The two peptides have synergistic activities and work in a 1:1 ratio

Each of the separated Plw peptides had low bacteriocin activity, but the titre of a mixture of them was higher than the sum of the individual contributions. Thus Plw $\alpha$  and Plw $\beta$  acted synergistically. The bacteriocin titres of the mixtures depended on the concentrations of both components. The relationship between mixing ratio and bacteriocin activity was studied in the microtitre plate assay with *Lb. sakei* NCDO 2714 as the indicator



**Fig. 4.** Bacteriocin activity of mixtures of Plw $\alpha$  and Plw $\beta$ . Bacteriocin activity was assayed in the microtitre plate assay, using *Lb. sakei* NCDO 2714 as the indicator strain. (a) Mixtures containing 7.5 BU Plw $\beta$  ml<sup>-1</sup> and varying amounts of Plw $\alpha$ . (b) Mixtures containing 80 BU Plw $\alpha$  ml<sup>-1</sup> and varying amounts of Plw $\beta$ .

organism. The effects of varying the mixing ratio in mixtures containing constant amounts of either component are shown in Fig. 4. In both experiments, the dose-response relationships appeared to be linear. This indicates that the activities of the mixtures can be described by the following formula:  $BU_{\alpha\beta} = BU_{\alpha} + BU_{\beta} + k \cdot (BU_{\alpha} \cdot BU_{\beta})$  or, more generally,  $BU_{\alpha\beta} = k_{\alpha} \cdot [\alpha] + k_{\beta} \cdot [\beta] + k_{\alpha\beta} \cdot [\alpha] \cdot [\beta]$ , where  $BU_{\alpha}$  is the activity of Plw $\alpha$  (in BU ml<sup>-1</sup>) alone,  $BU_{\beta}$  the activity units (ml<sup>-1</sup>) of Plw $\beta$  alone, and  $BU_{\alpha\beta}$  activity units (ml<sup>-1</sup>) of the mixture.  $k_{\alpha}$ ,  $k_{\beta}$  and  $k_{\alpha\beta}$  relate activity to the concentration of Plw $\alpha$ , Plw $\beta$  and mixtures, respectively.

The value of  $k$  was found to be 28 ml BU<sup>-1</sup> and 60 ml BU<sup>-1</sup> in the experiments with Plw $\alpha$  and Plw $\beta$  in excess,

respectively. Thus, great differences in the mixing ratio appear to have little effect on  $k$ .

## DISCUSSION

We describe here a new bacteriocin from *Lb. plantarum*. Strains of *Lb. plantarum* are used extensively in food fermentation, and the production of bacteriocins with broad inhibitory spectra enhances the potential for the application of these bacteria to food preservation (Daeschel & Nes, 1994). Plw is a two-peptide lantibiotic and has several properties previously not seen in bacteriocins.

Two-peptide bacteriocins have been isolated from many Gram-positive bacteria, in lactic acid bacteria in particular, and they are found amongst lantibiotics as well as non-lantibiotics. In most cases, it has been found that one of the two peptides exerts bacteriocin activity on its own, and that this activity is stimulated by the presence of the other peptide (Allison *et al.*, 1994; Anderssen *et al.*, 1998; Jiménez-Díaz *et al.*, 1995; Marciset *et al.*, 1997; Navaratna *et al.*, 1998; Ryan *et al.*, 1999). Neither of the two peptides of lactococcin G has any bacteriocin activity (Nissen-Meyer *et al.*, 1992). In the Plw system, however, both peptides had inherent antimicrobial activities. In two-peptide bacteriocin systems, this has previously been described only for enterocin L-50, the two peptides of which are almost identical (Cintas *et al.*, 1998). In this regard, Plw is different from the related bacteriocins staphylococcin C55 and lactacin 3147 in that it is only the Plw $\alpha$  homologue that shows inhibitory activity on its own (Navaratna *et al.*, 1998; Ryan *et al.*, 1999).

The relationship established between the concentrations of each of the two Plw peptides in a mixture and the mixture's antimicrobial activity indicates that the two peptides cooperate in a 1:1 ratio. A 1:1 ratio between the two peptides has been reported to be optimal for several two-peptide bacteriocins – lantibiotics as well as non-lantibiotics (Navaratna *et al.*, 1998; Moll *et al.*, 1996; Marciset *et al.*, 1997; Anderssen *et al.*, 1998). This stoichiometry has usually been inferred from the mixing ratio giving the highest specific bacteriocin activity. At suboptimal mixing ratios, the dose–response relationships have been found to differ significantly from that of the Plw system. In the case of staphylococcin C55, stimulation of the activity of the  $\alpha$  peptide was observed only in mixtures with mixing ratios ( $\beta/\alpha$ ) between 0.1 and 1 (Navaratna *et al.*, 1998). The enhancing factor, ThmB, of thermophilin 13 increased the activity of ThmA about 40-fold when the mixing ratio was 1, but an excess of ThmB was inhibitory to the bacteriocin activity (Marciset *et al.*, 1997). In the Plw system, on the other hand, an increasing and continuous response to the concentration of either peptide was seen, and the mixtures' activities were directly related to their concentrations. The relationship between peptide concentrations and bacteriocin activity suggests that the activity was limited by each peptide's affinity for its

target, and that the concentration of the other peptide had no or little influence on this affinity.

In lantibiotics, most Cys, Ser and Thr residues have undergone post-translational modifications, forming specific Lan/MeLan bridges and, usually,  $\alpha,\beta$ -unsaturated amino acids. By comparing the amino acid contents of the isolated peptides with those expected from the DNA sequences, it was clear that most of the Ser, Thr and Cys residues in Plw were modified. However, unmodified Cys was found in both of the Plw peptides: one residue was in Plw $\beta$  and two were in Plw $\alpha$ . In lantibiotics, unmodified Cys residues have previously been demonstrated only in sublancin 168 (Paik *et al.*, 1998). Furthermore, all of the Ser and Thr residues of Plw $\beta$  were modified, and our data indicate the presence of three  $\alpha,\beta$ -unsaturated amino acid residues and three Lan/MeLan cross-links. The number of thioether bridges appears to be the same in Plw $\alpha$ , but this peptide contained no  $\alpha,\beta$ -unsaturated amino acid residues and, more remarkably, an unmodified Ser residue. This is the first example of the presence of both a Cys and a Ser in a lantibiotic.

A number of two-peptide bacteriocins have been found in lactic acid bacteria; they show a remarkable diversity of primary structure. The two peptides of Plw show significant sequence similarities with the corresponding peptides of two other two-component lantibiotics, namely lactacin 3147 (from *Lc. lactis*) and staphylococcin C55 (from *S. aureus*) (Navaratna *et al.*, 1999; Ryan *et al.*, 1999). We suggest that these three bacteriocins constitute a new family of bacteriocins. They belong to the type A lantibiotics, which are linear peptides, as opposed to the circular type B lantibiotics (Jung, 1991). The type A lantibiotics have been grouped into two classes, namely class AI and class AII (de Vos *et al.*, 1995). This classification is mainly based on the properties of the leader peptide and the mechanism by which the bacteriocin is exported. Class AII lantibiotics are all translated with leader peptides of the so-called 'double-glycine' type, characterized by two conserved glycine residues at positions  $-1$  and  $-2$ . This kind of leader is also prevalent in non-lantibiotic peptide bacteriocins from Gram-positive and Gram-negative bacteria (Håvarstein *et al.*, 1994). The export of bacteriocins containing double-glycine-type leaders involves a dedicated ABC transporter containing an N-terminal proteolytic domain which cleaves off the leader peptide (Håvarstein *et al.*, 1995). The leader peptides of the bacteriocins exported by these ABC transporters are characterized by consensus elements occurring at defined distances relative to the cleavage site (Håvarstein *et al.*, 1995). The *plw* $\alpha$ A gene encodes a peptide of 59 aa, and the sequence of the N-terminal extension has most of the characteristics of a leader peptide of the double-glycine type. On the other hand, the amino acid sequence preceding the N-terminal residue of mature Plw $\beta$  did not conform to such a leader. With one exception (staphylococcin C55 $\beta$ ; Navaratna *et al.*, 1999) (Fig. 3), the Gly at position  $-2$  relative to the processing site is present in all of the leader peptides of the Gly-Gly type



**Fig. 5.** Proposed primary structures for Plw $\alpha$  (a) and Plw $\beta$  (b). Abu,  $\alpha$ -aminobutyric acid; Abu-S-Ala, 3-methylanthionine; Ala-S-Ala, lanthionine; Dha, 2,3-didehydroalanine; Dhb, 2,3-didehydrobutyrine; Pyr, pyruvic acid derived from deamination of Dha.

studied to date, and cleavage after the sequence Ala-Arg would be a violation of this rule. However, genes encoding ABC transporters with the characteristics of transporters of bacteriocins with double-glycine leaders are found downstream of the structural genes for lactacin 3147 (Dougherty *et al.*, 1998) and staphylococcin C55 (Navaratna *et al.*, 1999), as well as Plw (GenBank accession number AY007251). This suggests that these bacteriocins are indeed class AII lantibiotics. In the Plw $\beta$  prepeptide, an alternative processing site could be identified between positions  $-6$  and  $-7$  (Fig. 3). Thus, the sequence encompassing the first 29 residues in the Plw $\beta$  prepeptide could constitute a putative leader peptide of the double-glycine type. With such a leader peptide, the processing site would be after the sequence Gly-Ala, and eight of the nine consensus elements for double-glycine leader peptides would be present. The only consensus element missing is a hydrophobic residue at position  $-4$ , and this element is also absent from the leader peptides of lactococcin G $\alpha$  and the lantibiotic sublancin 168 (Paik *et al.*, 1998). We suggest, therefore, that Plw $\beta$  is synthesized with a 29 aa leader, processed by an ABC transporter and then subjected to further proteolytic attack, resulting in the removal of the six N-terminal residues. A similar situation is seen with plantaricin A. The sequence of isolated plantaricin A indicates that a few amino acid residues are removed from the N-terminus after cleavage of the prepeptide at the Gly-Gly processing site (Diep *et al.*, 1994).

The thioether bridging patterns have been determined for several lantibiotics, and homologous lantibiotics have conserved bridging patterns (Jack *et al.*, 1998). Also, the few engineered variants altered at the sites involved in ring formation showed little or no bacteriocin activity (Kuipers *et al.*, 1996). This indicates an important role for Lan/MeLan in lantibiotic function. However, the thioether bridging pattern has not been established for any two-peptide lantibiotic.

The enzymic formation of dehydrated amino acid residues and that of thioether bonds are specific processes. For unknown reasons, the thioether bonds in type A lantibiotics are formed between Cys residues and Ser- or Thr-derived residues located N-terminally to their partners (de Vos *et al.*, 1995; Jack *et al.*, 1998). This restricts the number of potential thioether bridges in the Plw peptides. The model presented in Fig. 5 shows a Lan cross-link between residues 8 and 18 in Plw $\alpha$ . As

expected, the unmodified Ser residue is located close to the C-terminus and is not conserved within the family. The model also shows two overlapping thioether bridges in the C-terminal part of the Plw $\alpha$  molecule. We consider this structure to be more likely than the alternative, which has one ring enclosed within the other – a pattern never seen in a type A lantibiotic. Furthermore, overlapping thioether bridges are found in most type A lantibiotics with a known bridging pattern (Jack *et al.*, 1998). The exceptions are lactocin S (Skaugen *et al.*, 1994), which has two bridges, and, of course, sublancin, which has only one bridge (Paik *et al.*, 1998).

The fact that the N-terminal residue of Plw $\beta$  was not accessible for Edman degradation indicates that the residue was deaminated. A dehydrated Ser residue located in this position would be unstable and would undergo spontaneous deamination, as in Pep5 (Kaletta *et al.*, 1989), epicidin 280 (Heidrich *et al.*, 1998), epilancin K7 (van de Kamp *et al.*, 1995) and lactocin S (Skaugen *et al.*, 1994). Thus, as in Plw $\alpha$ , none of the three thioether rings in Plw $\beta$  is located in the N-terminal part of the molecule. The assumptions mentioned above leave us with a Lan linking residues 14 and 18 and two thioether bridges in the region spanning residues 20–27. The region contains three residues derived from Thr or Ser, but only the bridging pattern illustrated in Fig. 5 gives overlapping ring structures.

The high numbers of hydroxy amino acid residues and Cys residues in the lactacin 3147 and staphylococcin C55 propeptides make it impossible to predict their bridging patterns directly from their sequences. Like Plw, they have three thioether bridges in each of their peptides (Navaratna *et al.*, 1998; Ryan *et al.*, 1999). Furthermore, the residues conserved among all three bacteriocins are mainly restricted to those that can be involved in thioether bridge formation. These findings, coupled with the fact that thioether bridges appear to be of vital importance for function, strongly suggest that the bridging patterns have been conserved within the family. Thus, the thioether bridging patterns suggested in Fig. 5 also apply to lactacin 3147 and staphylococcin C55. However, the cystine is unique to the Plw $\alpha$  molecule. The overlapping cross-links give the Plw $\alpha$  molecule two rigid parts joined by the peptide bond between residues 18 and 19. The corresponding peptides of staphylococcin C55 and lactacin 3147 would lack the cystine bridge and would have more-flexible N-terminal parts. Although

reduction of the cystine bridge increased the activity of isolated Plw $\alpha$  about fourfold, it had no effect on the activities of mixtures of Plw $\alpha$  and Plw $\beta$ . Several type A lantibiotics have been found to form pores in bacterial cytoplasmic membranes. It has been suggested that the peptides remain surface bound during the pore-formation process and enter the membrane in a bent conformation (van den Hooven *et al.*, 1996a, b). The peptides contain a central hinge region, and it has been shown for nisin, epidermin and Pep5 that this flexible region is essential for activity (Sahl *et al.*, 1995). Apparently, such a flexible region is not important for Plw activity.

As discussed above, we suggest that the bridging patterns shown are common to all three members of this new family of two-peptide lantibiotics. We have taken advantage of the low sequence similarities between Plw and its relatives to pinpoint residues contributing to structural rigidity. Our data have enabled us to present the first model for the localization of thioether cross-links in two-peptide lantibiotics; these data will also be useful in elucidating the structure–function relationships of such antimicrobial compounds.

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