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Plantaricyclin A, a Novel Circular Bacteriocin Produced by *Lactobacillus plantarum* NI326: Purification, Characterization, and Heterologous Production

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ABSTRACT Bacteriocins from lactic acid bacteria (LAB) are of increasing interest in recent years due to their potential as natural preservatives against food and beverage spoilage microorganisms. In a screening study for LAB, we isolated from olives a strain, *Lactobacillus plantarum* NI326, with activity against the beverage-spoilage bacterium *Alicyclobacillus acidoterrestris*. Genome sequencing of NI326 enabled the identification of a gene cluster (designated *plc*) encoding a putative circular bacteriocin and proteins involved in its modification, transport, and immunity. This novel bacteriocin, named plantaricyclin A (PlcA), was grouped into the circular bacteriocin subgroup II due to its high degree of similarity with other gassericin A-like bacteriocins. Purification of PlcA from the supernatant of *Lb. plantarum* NI326 resulted in an active peptide with a molecular mass of 5,570 Da, corresponding to that predicted from the (processed) PlcA amino acid sequence. The *plc* gene cluster was cloned and expressed in *Lactococcus lactis* NZ9000, resulting in the production of an active 5,570-Da bacteriocin in the supernatant. PlcA is believed to be produced as a 91-amino-acid precursor with a 33-amino-acid leader peptide, which is predicted to be removed, followed by joining of the N and C termini via a covalent linkage to form the mature 58-amino-acid circular bacteriocin PlcA. We report the characterization of a circular bacteriocin produced by *Lb. plantarum*. The inhibition displayed against *A. acidoterrestris* highlights its potential use as a preservative in food and beverages.

IMPORTANCE In this work, we describe the purification and characterization of an antimicrobial peptide, termed plantaricyclin A (PlcA), produced by a *Lactobacillus plantarum* strain isolated from olives. This peptide has a circular structure, and all genes involved in its production, circularization, and secretion were identified. PlcA shows antimicrobial activity against different strains, including *Alicyclobacillus acidoterrestris*, a common spoilage bacterium, which causes substantial economic losses in the beverage industry every year. In this study, we describe a circular antimicrobial peptide, PlcA, for a *Lactobacillus plantarum* strain.

KEYWORDS circular bacteriocin, *Alicyclobacillus acidoterrestris*, *Lactobacillus plantarum*, immunity

Bacteriocins are ribosomally synthesized antimicrobial peptides produced by bacteria to inhibit the growth of other, often closely related, strains (1). Bacteriocin production is a common feature among food-grade lactic acid bacteria (LAB), and bacteriocins have, for this reason, attracted considerable interest for their potential use as natural and nontoxic food preservatives (2, 3). Some of these peptides have

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demonstrated greater efficacy than conventional antibiotics against numerous pathogenic and drug-resistant bacteria, while not displaying any toxicity toward eukaryotic cells (4). For this reason, bacteriocins may also be useful in human and veterinary applications as a powerful weapon in the ongoing battle against antibiotic resistance, including for the treatment of local and systemic bacterial infections (4–6).

Within the different families of bacteriocins, circular bacteriocins constitute a unique group of active proteins in which the N- and C-terminal ends are covalently linked to form a circular backbone (7). This additional bond is thought to enhance the thermodynamic stability and structural integrity of the peptide and consequently improve its biological activity (8–10). To date, only a small number of circular bacteriocins have been described. These can be subdivided in two major groups according to their physicochemical characteristics and level of sequence identity (10). Subgroup I encompasses circular bacteriocins with a high content of positively charged amino acids and a high isoelectric point (pI, ~10). This includes the best-studied circular bacteriocin, enterocin AS-48 (11), together with other bacteriocins, such as carnocyclin A (12), circularin A (13), lactocyclin Q (14), and garvicin ML (15). Subgroup II circular bacteriocins include bacteriocins with a smaller number of positively charged amino acid residues and a medium to low isoelectric point (pI, between ~4 and 7). Currently this group comprises just three members, gassericin A (16), butyrivibriocin AR10 (17), and acidocin B (18).

In this study, we screened 50 colonies, isolated from olives, for their potential to inhibit growth of the beverage-spoilage strain *Alicyclobacillus acidoterrestris* sp1. We report the purification and genetic characterization of a circular gassericin A-like bacteriocin, termed plantaricyclin A, produced by *Lactobacillus plantarum* NI326, with antimicrobial activity against various microorganisms, including *A. acidoterrestris* sp1.

RESULTS AND DISCUSSION

Alicyclobacillus acidoterrestris is considered to be one of the species with the highest food spoilage impact worldwide (19). *A. acidoterrestris* is a thermoacidophilic spore-forming bacterium with a strong spoiling potential, especially in low-pH juices. The presence of *A. acidoterrestris* in juices is difficult to detect visually, but its presence is associated with an unpleasant odor caused by the production of guaiacol and other halophenols by the strain. Bacteriocins, such as the lantibiotic nisin A or the circular bacteriocin enterocin AS-48, have shown some promising results when used as strategies to inhibit growth of *A. acidoterrestris* in juices (20, 21).

Isolation and identification of *Lactobacillus plantarum* NI326. In this study, we screened 50 presumed LAB isolates from olives with the aim of finding an isolate exhibiting antimicrobial activity against *A. acidoterrestris* sp1. Only one out of the 50 obtained isolates was shown to exhibit a zone of inhibition against the indicator strain. This colony was identified as *Lb. plantarum* by 16S rRNA sequencing and designated *Lb. plantarum* NI326. No zone of inhibition was apparent when the cell-free culture supernatant (CFS) was first treated with proteinase K, confirming the proteinaceous nature of the antimicrobial compound (data not shown).

Genome sequence analysis of *Lb. plantarum* NI326. To find potential bacteriocin-encoding gene clusters, the genome of *Lb. plantarum* NI326 was sequenced, generating 84 contigs following sequence assembly. *In silico* analysis of the 84 contigs with BAGEL3 detected a potential bacteriocin gene cluster (designated here as *plc*) predicted to encode a peptide with a 43-amino-acid (aa) putative conserved domain corresponding to the subgroup II gassericin A-like circular bacteriocins. This putative peptide, designated plantaricyclin A (PlcA), exhibits 67% similarity to the circular bacteriocin gassericin A. An alignment of this peptide with all other members of the gassericin A-like circular bacteriocin group, gassericin A (GaaA), acidocin B (AciB), and butyrivibriocin AR10 (BviA), revealed a high degree of similarity. This alignment facilitated the prediction of the cleavage site of the signal peptide from the mature peptide to be between amino acids N33 and I34 (Fig. 1). Both GaaA and AciB are synthesized as 91-aa prepeptides with 33-aa leader peptides that are cleaved off, followed by a covalent

A)

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GaaA  MVTKYGRNLGLNKVELFAIWAVLVVALLLTTAN IYWIADQFGIHLATGTARKLLDAMASGASLGTAFAAILGVTLPAWALAAAGALGATAA 91
AciB  MVTKYGRNLGLSKVELFAIWAVLVVALLLATAN IYWIADQFGIHLATGTARKLLDAVASGASLGTAFAAILGVTLPAWALAAAGALGATAA 91
BviA            MSKKQIMSNCISIALLIALIPN IYFIADKMGIQLAPAWYQDIVNWVSAGGTLTTGFAIIVGVTVPAWIAEAAAAFGIASA 80
PlcA  MLSAYRSKLGLNKFEVTVLMIISLFILLFATVN IVWIAKQFGVHLTTSLTQKALDLLSAGSSLGTVAAAVLGVTLPAWAVAAAGALGGTAA 91
                :.* ::     : :  *:   * * :**.::*::*: .  :. :: :::*.:* *  * ::***:***   **.*:* ::*
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B)

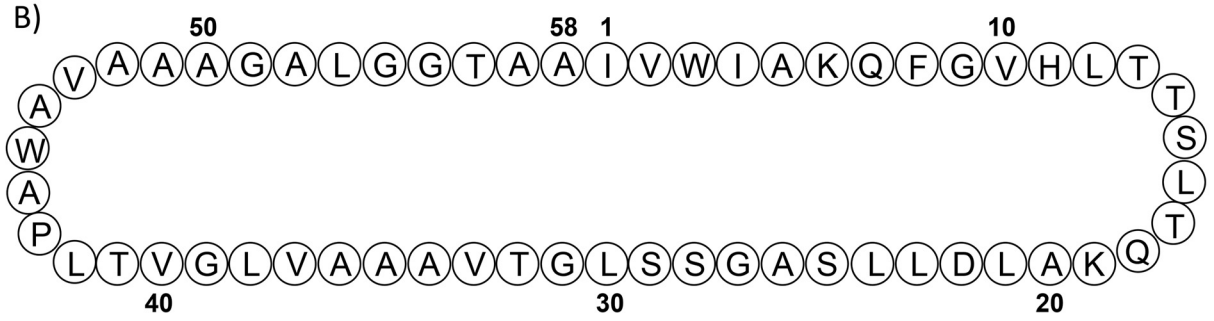

FIG 1 (A) Sequence alignment of all the members of subgroup II circular bacteriocins with plantaricyclin A, using MUSCLE (41). Conserved, conservative, and semiconservative substitutions are indicated by asterisks, colons, and semicolons, respectively. Bold letters depict the leader sequence. (B) Schematic plantaricyclin A mature peptide.

linkage between the N and C terminus, to form the mature 58-aa circular bacteriocin. In previous studies, sequence alignments between characterized and hypothetical subgroup II circular bacteriocins did reveal the presence of a fully conserved asparaginyl cleavage site (18), which is also present in PlcA.

The function of these leader peptides and mechanism through which peptide circularization occurs is still unclear. One of the biggest challenges in the field of circular proteins is finding out how their ends are stitched together from their linear precursors (22). Identification of this mechanism has the potential to facilitate the creation of new, highly stable antimicrobial agents for use in food, veterinary, and medical applications (12). PlcA has a predicted mass of 5,588 Da and represents a new bacteriocin within the subgroup II and the first (predicted) circular bacteriocin isolated from *Lb. plantarum*.

Analysis of the *plc* gene cluster revealed the presence of seven open reading frames (ORFs) downstream of the PlcA-encoding gene (*plcA*), with sequence and organizational similarity to those found in the gene clusters responsible for GaaA and AciB production (Table 1 and Fig. 2). Accordingly, *plcA* is followed by *plcD*, which encodes a putative 157-aa membrane-associated protein with a DUF95 conserved domain. Recent research suggests that DUF95 proteins play a dual role in the biosynthesis of circular peptides, as an immunity-associated transporter protein and as a secretion-aiding agent (23). The ORF, which is designated *plcI*, is located immediately downstream of *plcD*, and encodes a 54-aa protein with a hypothetical function as an immunity protein. Kawai et al. (24) showed that heterologous expression of GaaI, which is similar to the *plcI* protein product, in *Lactococcus lactis* confers a 7-fold-higher resistance to gassericin A compared to that of a control strain.

The next two genes of the cluster (*plcT* and *plcE*) encode proteins of 227 aa and 214 aa, respectively (Table 1). Both have conserved ATP-binding domains linked to proteins

TABLE 1 Putative proteins derived from the *plc* operon

ORF	Product length (aa)	Amino acid identity (%) relative to gassericin A gene cluster homologs	Hypothetical function
plcA	90	56	Plantaricyclin A precursor
plcD	157	33	Unknown, DUF95 family
plcI	54	33	Immunity
plcT	227	45	ATP-binding protein
plcE	214	37	Membrane transporter
plcB	173	30	Unknown
plcC	56	35	Unknown

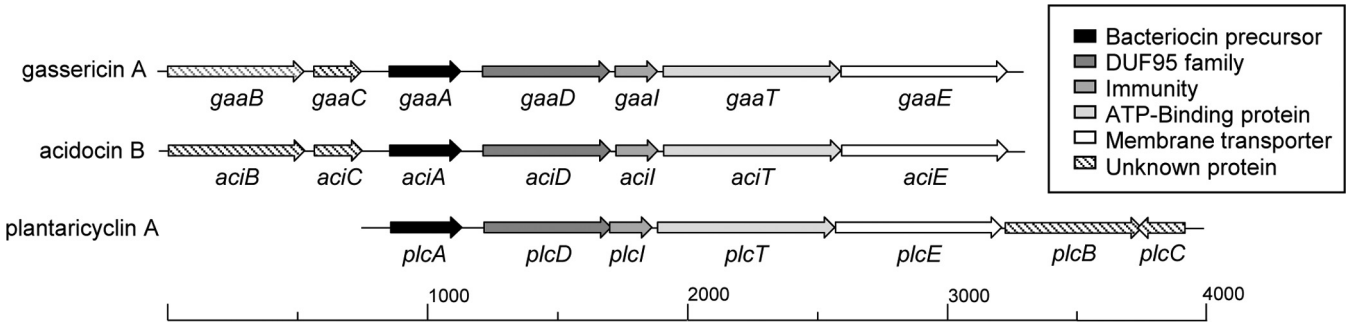

FIG 2 Schematic representation of the gene clusters involved in the production of the circular bacteriocins gassericin A (24), acidocin B (18) and plantaricyclin A. The known or putative biochemical function or properties are denoted by color, as indicated in the key.

of the ABC transporter family, and based on homology to their equivalents from the gassericin A and acidocin B associated gene clusters, they are most likely involved in the secretion of PlcA. The downstream *plcB* and *plcC* genes are in positions that are different from their homologs in the clusters for GaaA and AciB production (Fig. 2). The function of the proteins coded by these two genes is still unknown, but their presence in all of the clusters from circular bacteriocins clearly indicates that they play an important role (9).

Heterologous production of PlcA in *L. lactis* NZ9000. To further confirm that PlcA is responsible for the activity shown by *Lb. plantarum* NI326, the entire *plc* cluster was cloned into the nisin-inducible plasmid pNZ8048 (pNZPlc) and transformed into *L. lactis* NZ9000, a naturally non-bacteriocin-producing strain. The CFS from *L. lactis* pNZPlc was shown to exhibit antimicrobial activity against *A. acidoterrestris* sp1 similar to that from the wild-type *Lb. plantarum* NI326 (Fig. 3A). The production of PlcA by *L. lactis* confirms that the cluster contains all necessary information for the correct production, modification, and secretion of PlcA. Based on these results and the similarity of the *plc* cluster to those from GaaA and AciB, we hypothesize that the biosynthetic machinery for all members of this bacteriocin subgroup is similar.

Analysis of immunity to PlcA. In order to determine if *plcD* and/or *plcI* encode immunity proteins for PlcA, the genes were cloned individually or together in the NisA-inducible vector pNZ8048 and transformed into *L. lactis* NZ9000. The recombinant strain *L. lactis* NZ9000(pNZPlcDI) induced with nisin A displayed full resistance to PlcA, while strains *L. lactis* NZ9000(pNZPlcD) and *L. lactis* NZ9000(pNZPlcI) induced with NisA

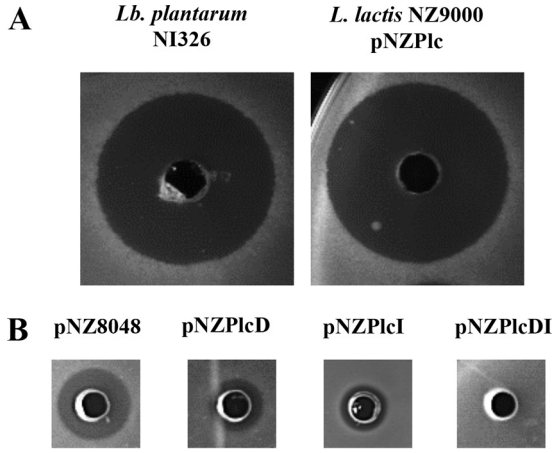

FIG 3 (A) Antimicrobial activity of the CFS of *L. plantarum* NI326 and nisin A-induced *L. lactis* NZ9000(pNZPlc) against *A. acidoterrestris* sp1. (B) Antimicrobial activity of the CFS of *Lb. plantarum* NI326 against cultures of *L. lactis* NZ9000(pNZ8048), *L. lactis* NZ9000(pNZPlcD), *L. lactis* NZ9000(pNZPlcI), and *L. lactis* NZ9000(pNZPlcDI) uninduced (−) or induced (+) with nisin A.

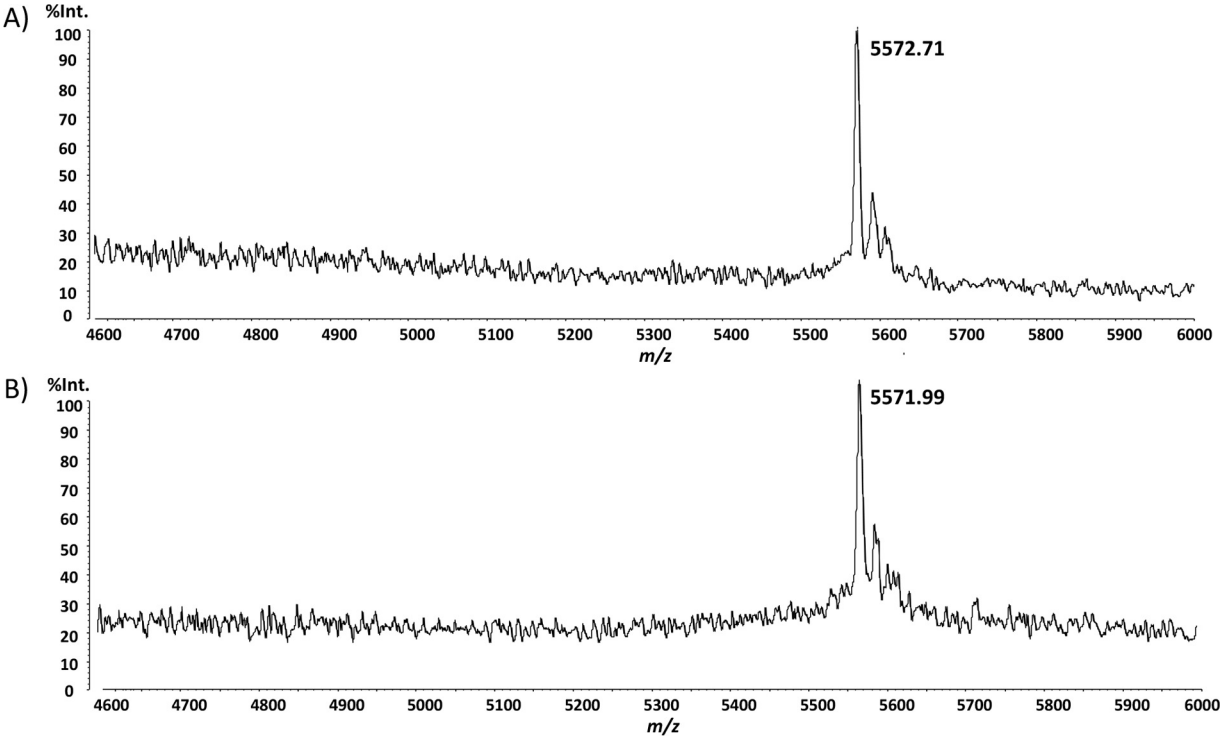

FIG 4 MALDI-TOF mass spectrometry analysis of the purified plantaricyclin A produced by *L. lactis* pNZPlca (A) and *L. plantarum* NI326 (B). %Int., percentage of intensity.

still exhibited sensitivity to PlcA, but at a visibly lower level compared to the control strain *L. lactis* NZ9000(pNZ8048) (Fig. 3B). Therefore, although both proteins individually appear to confer partial immunity to *L. lactis* NZ9000 against the antimicrobial activity of PlcA, the recombinant strain was fully protected against the action of PlcA when both proteins were being produced concomitantly. Similar results have been observed with other circular bacteriocins, such as carnocyclin A, where the production of the immunity protein (CclI) was not enough to confer full protection to the producer, and only when CclD and CclI were coproduced did the strain show full immunity (25).

Purification and MALDI-TOF analyses of the antimicrobial activity of *Lb. plantarum* NI326. The antimicrobial peptide produced in the CFS by *Lb. plantarum* NI326 and *L. lactis* pNZPlc was purified by using reversed phase high-performance liquid chromatography (RP-HPLC), and the molecular mass was analyzed by using matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS). In both cases, a single mass of 5,572 Da was detected in the active fractions (Fig. 4). The 18-Da difference between the molecular mass of PlcA and its theoretical mass calculated from the AA sequence corresponds to the loss of a molecule of water that occurs during circularization of the peptide, as reported for other circular bacteriocins (18, 26).

Sensitivity of plantaricyclin A to heat, pH, and proteolytic enzymes. The antimicrobial activity of partially purified PlcA was the same as the initial antagonistic activity following exposure to temperatures ranging from 30°C to 100°C for 10 min, suggesting the relative stability of the bacteriocin. No antimicrobial activity was lost when PlcA was adjusted to pH values 2 to 10. The antimicrobial activity of PlcA was completely lost when treated with proteinase K and pronase, whereas pepsin and α-chymotrypsin treatments resulted in the retention of 100% and 78% of the initial antagonistic antimicrobial activity, respectively (results not shown).

The resistance of circular bacteriocins to temperature, pH variations, and proteolytic enzymes is due mainly to their three-dimensional conformation. The solution structure of acidocin B has recently been solved. Accordingly, AciB is composed of four α-helices of similar length folded to form a compact, globular bundle that allows the formation

TABLE 2 Strains used in this study, sources, and activity of PlcA

Strain	Source[a]	Activity[b]
Alicyclobacillus acidoterrestris sp1	Coca Cola Co.	+
Lactococcus lactis HP	UCC	+
L. lactis KH	UCC	+
L. lactis MG1363	UCC	+
L. lactis RT28	UCC	+
L. lactis NZ9000	UCC	+
Lactobacillus bulgaricus UCC	UCC	+
Lactobacillus plantarum PARA	UCC	−
L. plantarum WCFSI	UCC	−
Lactobacillus brevis MB124	UCC	−
L. brevis SAC12	UCC	−
L. brevis L102	UCC	−
L. brevis L94	UCC	−
Pediococcus claussenii H5	UCC	−
Pediococcus inopinatus 1011	UCC	+
Enterococcus faecium DPC1146	UCC	−
Listeria innocua UCC	UCC	−
Listeria monocytogenes EGD-e	UCC	−
L. monocytogenes 33077	UCC	−
Escherichia coli EC10B	UCC	−
Staphylococcus aureus DPC5243	UCC	−
Streptococcus uberis ATCC 700407	UCC	−
Streptococcus dysgalactiae GrpC	UCC	−
Salmonella enterica serovar Typhimurium UTC1lux	UCC	−
Klebsiella pneumoniae UCC	UCC	−
Bacillus cereus DPC6087	UCC	−

[a]UCC, University College Cork.
[b]+, zone of inhibition observed; −, no zone of inhibition observed.

of a central pore, resembling the structure of the saposins. The surfaces of acidocin B and gassericin A are dominated by hydrophobic and uncharged residues and, therefore, it is believed that the initial contact between these circular peptides and the target strains is mediated by hydrophobic interactions (18).

Antimicrobial spectrum of plantaricyclin A. Aliquots of HPLC purified fractions of PlcA were evaluated for their antimicrobial activities and inhibitory spectra against different indicator microorganisms. The strains *A. acidoterrestris* sp1, *Lb. bulgaricus* UCC, *Pediococcus inopinatus* 1011, and all tested lactococcal strains were inhibited by the bacteriocin produced by *Lb. plantarum* NI326 (Table 2).

In addition to the spectra of inhibition, we observed some other differences between PlcA and the other members of subgroup II, such as a higher isoelectric point (8.6) and a net charge of +1 (27). In fact, some authors use the pI values and net charges to differentiate between circular bacteriocins of subgroup I (pI, ~10 and positively charged) from circular bacteriocins of subgroup II (pI, 4 to 7 and uncharged or slightly negative) (10). According to this classification system, PlcA should be placed in an intermediate position between subgroups I and II. However, we strongly believe that this peptide should be classified within subgroup II and propose to modify the classification criteria and broaden the pI range for this subgroup to be between 4 and ~9.

Plantaricyclin A represents the first circular bacteriocin isolated and characterized from an *Lb. plantarum* strain. The antimicrobial activity observed against the food and beverage spoilage microorganism *Alicyclobacillus acidoterrestris* should be further studied, as this strain represents a significant problem for the food industry. The use of bacteriocins, such as nisin A and enterocin AS-48, as preservatives in low-pH beverages and juices has shown some promising results to control the growth of *A. acidoterrestris* (28). The circular nature of PlcA makes it especially interesting for industrial applications, as this peptide could survive and retain most of the activity under changing conditions (temperature and pH, for example) during food/beverage manufacture. Moreover, the narrow spectrum of activity from PlcA can be considered an advantage,

especially in fermented beverages. In comparison to other broad-spectrum bacteriocins, such as nisin A or enterocin AS-48, PlcA could be used to specifically target *A. acidoterrestris* spp., while having little or no effect against other desirable microorganisms present in the beverage. However, a more detailed investigation, including that of more indicator strains and other *A. acidoterrestris*, is needed in order to further assess the full potential and applicability of this biofunctional peptide.

MATERIALS AND METHODS

Cultures and growth conditions. The strains used in this study are summarized in Table 2. All *Lactobacillus*, *Pediococcus* and *Leuconostoc* strains were grown in de Man-Rogosa-Sharpe (MRS) medium (Oxoid, Hampshire, United Kingdom) at 30°C, *A. acidoterrestris* sp1 was grown in *Bacillus acidoterrestris* (BAT) broth (Pronadisa, Spain) at 45°C, while some of the other indicator strains were grown in LB broth (1% peptone, 1% NaCl, 0.5% yeast extract) at 37°C (*Escherichia coli*, *Salmonella enterica* serovar Typhimurium, and *Klebsiella pneumoniae*), brain heart infusion (BHI) broth (Oxoid) at 37°C (*Staphylococcus aureus*, *Listeria monocytogenes*, *Listeria innocua*, and *Bacillus cereus*), TSB broth (Oxoid) at 37°C (*Streptococcus uberis* and *Streptococcus dysgalactiae*) and M17 broth (Oxoid) supplemented with 0.5% glucose (Sigma-Aldrich, USA) at 30°C (*Lactococcus lactis*) or at 37°C (*Enterococcus faecium*). Chloramphenicol (Sigma-Aldrich) was added at 5 μg/ml where required. All of these microorganisms were grown under aerobic conditions. All strains were stored at −80°C in their respective media with 20% glycerol until required for use.

Isolation of LAB strains from olives. Over 50 isolates were isolated from olives as previously described (29). Briefly, 5 g of olives (obtained from the English Market, Cork, Ireland) was homogenized with 45 ml of Ringer's solution using a stomacher at 300 bpm for 1 min (stomacher circular 400; Seward, UK). The resulting homogenate was serially diluted in Ringer's solution, and 100 μl of each dilution plated on MRS agar (Oxoid) plates supplemented with 100 μg/ml cycloheximide (Sigma) to suppress fungal growth. Plates were then incubated at 30°C anaerobically for 2 days. Colonies obtained were handpicked and inoculated into 250-μl aliquots of MRS broth in 96-well plates. Cultures were grown anaerobically overnight at 30°C and stored at −80°C with 20% glycerol for further analysis.

Isolation of anti-*A. acidoterrestris* sp1 bacteriocin-producing LAB. Presumed LAB isolates exerting antimicrobial activity were identified using the spot-on-lawn method (29). Briefly, 5-μl aliquots of LAB cultures were spotted onto MRS agar plates and grown at 30°C anaerobically for 48 h. Plates were then overlaid with 5 ml of MRS soft agar (MRS broth supplemented with 0.8% bacteriological agar) seeded with 10^5 to 10^6 CFU/ml of an overnight culture of *L. lactis* HP. Plates were incubated at 30°C for 48 h, after which zones of inhibition surrounding the LAB colony were measured.

The LAB isolate showing inhibition against *L. lactis* HP was further cultured in 10 ml MRS broth and grown at 30°C overnight. Cell-free culture supernatant (CFS) was obtained by centrifugation of the culture at 12,000 × *g*, 4°C for 10 min and filtered through 0.2-μm-pore-size filters (Whatman International Ltd., Maidstone, UK). The activity of the CFS against *A. acidoterrestris* sp1 was analyzed using an agar diffusion test (ADT) (30). Briefly, 100-μl aliquots of CFS were placed in wells (6-mm diameter) bored in cooled *Alicyclobacillus* agar (Pronadisa) plates (30 ml) previously seeded (10^5 CFU/ml) with *A. acidoterrestris* sp1. Plates were incubated at 50°C to allow growth of the target organism and checked for zones of inhibition after 24 to 48 h.

Identification of LAB isolates. Individual colonies were used as the templates for PCR. The primers Luc-F (5′ CTT GTT ACG ACT TCA CCC 3′) and Luc-R (5′ TGC CTA ATA CAT GCA AGT 3′) (Eurofins MWG, Ebersberg, Germany) were used to amplify a variable region of the 16S rRNA gene (31). The following conditions were used for the PCRs: 95°C for 60 s, 53°C for 60 s, and 72°C for 95 s for 30 cycles. The DNA from individually purified amplicons was subjected to Sanger sequencing (Eurofins MWG), and the corresponding species identity was obtained by comparative sequence analysis (BLASTN) against available sequence data in the National Center for Biotechnology Information (NCBI) database (32).

***Lactobacillus plantarum* NI326 genome sequencing, genome annotation, and bacteriocin screening.** The genome of *Lb. plantarum* NI326 was sequenced using a combined Roche GS-FLX Titanium and Illumina HiSeq 2000 approach (GATC Biotech, Constance, Germany) to a final coverage of ~490-fold. Sequences obtained were first quality checked using IlluQC.pl from the NGS QC Toolkit v2.3 (http://www.nipgr.res.in/ngsqctoolkit.html) (33) and assembled with AbySS v1.9.0 (34).

Following sequence assembly, the generated contigs were employed to perform open reading frame (ORF) prediction with Prodigal v2.5 prediction software (http://gensoft.pasteur.fr/docs/prodigal/2.50/_README), supported by BLASTX v2.2.26 alignments (35). ORFs were automatically annotated using BLASTP v2.2.26 (35) analysis against the nonredundant protein database curated by the NCBI database. Following automatic annotation, ORFs were manually curated using the Artemis v16 genome browser and annotation tool (http://www.sanger.ac.uk/science/tools/artemis). The software tool was used to inspect and validate ORF results, to adjust start codons where necessary, and to aid in the identification of pseudogenes. The resulting ORF annotations were further refined, where required, using the alternative databases Pfam (36) and Uniprot/EMBL (http://www.uniprot.org/). Transfer tRNA was predicted using tRNA-scan-SE v1.4 (37). The whole genome was analyzed with the Web-based bacteriocin genome mining tool BAGEL3 (http://bagel.molgenrug.nl/) (38) to search for known and/or potential novel bacteriocins.

Cloning of the *plc* gene cluster in *L. lactis* NZ9000. Primers, PCR fragments, and plasmids used in this study are listed in Table 3. All primers were ordered from Eurofins. Plasmid derivatives were constructed as follows: primers Plc-F/Plc-R were used for PCR amplification of a 3,172-bp fragment from

TABLE 3 Primers, PCR products, and plasmids used in this study

Primer, PCR fragment, or plasmid	Nucleotide sequence (5′ to 3′) or description[a]
Primers (PCR fragment)	
Plc-F (Plc-Clust)	AACGCAAATGTTCCACACGG
Plc-R (Plc-Clust)	GGATTGGACTAGTAGCTCTAGGGT
NcoI-Plc (PlcADITEB)	CACTCACCATGGGTTAATGCTTTCAGCATATCGTAGTAAAT
XbaI-Plc (PlcADITEB)	ATCTATCTAGACTATAAAAAAATCAAGCTATATATAGG
NcoI-PlcD (PlcD/PlcDI)	CACTCACCATGGTGAATAAACCGCGGAGTAATATC
XbaI-PlcD (PlcD)	ATCTATCTAGATTAATCTCCTAACAACCATAAGGC
NcoI-PlcI (PlcI)	CACTCACCATGGTTGTTAGGAGATTAATTATGAAGAATTTAG
XbaI-PlcI (PlcI/PlcDI)	ATCTATCTAGATTAATCTGTATGCCGTTTAATTAGCTGA
pNZ-F	TGTCGATAACGCGAGCATAA
pNZ-R	CAAAGCAACACGTGCTGTAA
PCR fragments	
Plc-Clust	3,172-bp fragment external to Plc cluster
PlcADITEB	2,908-bp NcoI/XbaI fragment containing genes *plcA*, *plcD*, *plcI*, *plcT*, *plcE*, and *plcB*
PlcD	495-bp NcoI/XbaI fragment containing gene *plcD*
PlcI	204-bp NcoI/XbaI fragment containing gene *plcI*
PlcDI	662-bp NcoI/XbaI fragment containing genes *plcD* and *plcI*
Plasmids	
pNZ8048	Cm^r; inducible expression vector carrying the *nisA* promoter (42)
pNZPlc	pNZ8048 derivative containing PlcADITEB
pNZPlcD	pNZ8048 derivative containing PlcD
pNZPlcI	pNZ8048 derivative containing PlcI
pNZPlcDI	pNZ8048 derivative containing PlcDI

[a]Cleavage site for restriction enzymes is underlined. Cm^r, chloramphenicol resistance.

total genomic DNA of *Lb. plantarum* NI326, which encompassed the entire *plc* gene cluster, including its promoter(s). Using this *plc* gene cluster fragment as the template and the primer pairs NcoI-Plc/XbaI-Plc, NcoI-PlcD/XbaI-PlcD, NcoI-PlcI/XbaI-PlcI, and NcoI-PlcD/XbaI-PlcI, fragments encompassing *plcADITEB*, *plcD*, *plcI*, and *plcDI*, respectively, were amplified (Table 3). Such fragments were digested with NcoI and XbaI and ligated into pNZ8048, digested with the same enzymes. The ligation mixtures were used to transform *L. lactis* NZ9000 competent cells as previously described (39). The plasmid derivatives pNZPlc, pNZPlcD, pNZPlcI, and pNZPlcDI, were checked by colony-PCR and sequencing of the inserts using primers PNZ-F/PNZ-R (Table 3).

Purification and MALDI-TOF mass spectrometry analysis of PlcA. PlcA was purified from *Lb. plantarum* NI326 and *L. lactis* NZ9000 was transformed with pNZPlc, as described previously (40), with modifications. Briefly, a 1-liter CFS of *Lb. plantarum* NI326 was obtained as previously described. Recombinant *L. lactis* NZ9000(pNZPlc) was induced for the production of PlcA at an optical density at 600 nm (OD_{600}) of 0.5, using nisin A (Nisaplin; Dupont, USA) at a final concentration of 10 ng/ml. The induced culture was grown at 32°C for 3 h. CFS was obtained by centrifugation of the culture at 12,000 × *g* at 4°C for 10 min. Activity of the CFS from either strain against *A. acidoterrestris* sp1 was confirmed on an ADT, as previously described. CFS was applied to a 10-g (60-ml) Varian C_{18} bond elution column (Varian, Harbor City, CA) preequilibrated with methanol and water. The column was washed with 20% ethanol, and the inhibitory peptide was eluted in 100 ml of 70% 2-propanol 0.1% trifluoroacetic acid (TFA). Aliquots (15 ml) were concentrated to 2 ml through the removal of 2-propanol by rotary evaporation (Buchi). Samples were then applied to a semipreparative Vydac C_4 mass spectrometry (10 by 250 mm, 300 Å, 5 μm) RP-HPLC column (Grace, Columbia, USA) running an acetonitrile and propan-2-ol gradient described as follows: 5% to 55% buffer B and 0% to 5% buffer C over 25 min, followed by and 55% to 19% buffer B and 5% to 65% buffer C over 60 min, 19% to 5% buffer B and 65% to 95% buffer C over 5 min, where buffer A is Milli-Q water containing 0.1% TFA, buffer B is 90% acetonitrile 0.1% TFA, and buffer C is 90% propan-2-ol– 0.1% TFA. The eluent was monitored at 214 nm, and fractions were collected at 1-min intervals. Fractions were assayed on *Lactobacillus bulgaricus* (a highly sensitive strain) indicator plates and active fractions assayed for the antimicrobial mass of interest using MALDI-TOF mass spectrometry (MALDI-TOF MS). MALDI-TOF MS was performed with an Axima TOF² MALDI-TOF mass spectrometer (Shimadzu Biotech, Manchester, UK), as described by Field et al. (40).

Analysis of immunity against PlcA. The immunity of wild-type *L. lactis* NZ9000 and recombinant strains *L. lactis* NZ9000(pNZPlcD), *L. lactis* NZ9000(pNZPlcI), and *L. lactis* NZ9000(pNZPlcDI) was tested against the CFS from *Lb. plantarum* NI326 on an ADT assay as described above. The indicator strains were seeded in GM17– 0.8% agar with and without 10 ng/ml nisin A. The areas of zones of inhibition were measured after 24 h growth at 30°C. The absence of a zone indicates that the strain is immune to PlcA. Experiments were performed in triplicate to confirm the results.

Sensitivity of PlcA to heat, pH, and proteolytic enzymes. Aliquots of a PlcA-containing fraction obtained following reversed-phase HPLC were subjected to the following treatments: (i) 20-fold (vol/vol) dilution with 30% 2-propanol containing 0.1% TFA and heating at 80°C and 100°C for 30 min and at 121°C for 15 min to determine the stability of PlcA to heat; (ii) 20-fold (vol/vol) dilution in 10 mM Tris

buffer followed by a pH adjustment at 2, 3, 4, 5, 6, 7, 8, 9, and 10 with 1 M HCl or 1 M NaOH to evaluate the effect of pH on bacteriocin activity; and (iii) dilution as described in step ii, followed by the addition of α-chymotrypsin (Sigma), pepsin (Sigma), pronase (Sigma), and proteinase K (Sigma) at pH 7.0. Each enzyme was added to a final concentration of 1 mg/ml to determine PlcA sensitivity to proteolytic enzymes. After each treatment, the residual antimicrobial activity of PlcA was determined by the agar diffusion test (ADT) with *A. acidoterrestris* sp1 as the indicator microorganism. Experiments were performed in triplicate.

Antimicrobial spectrum of PlcA. Aliquots of 100 μl purified PlcA from *Lb. plantarum* NI326 were used to test its antimicrobial activity against various indicators (Table 2) using an ADT assay as described above. Experiments were performed in triplicate.

Accession number(s). The genome assembly of *Lb. plantarum* NI326 is deposited in GenBank under accession number GCA_002407395.1. The nucleotide sequences reported in this study are deposited in GenBank under accession numbers NDXC01000001.1 to NDXC01000084.1.

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