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Title	Plantaricyclin A, a novel circular bacteriocin produced by Lactobacillus plantarum NI326: purification, characterization, and heterologous production
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Publication date	2017-12-15
Original Citation	Borrero, J., Kelly, E., O'Connor, P. M., Kelleher, P., Scully, C., Cotter, P. D., Mahony, J. and van Sinderen, D. (2017) 'Plantaricyclin A, a novel circular bacteriocin produced by Lactobacillus plantarum NI326: purification, characterization, and heterologous production', Applied and Environmental Microbiology, 84(1), e01801-17 (10pp). doi:10.1128/aem.01801-17
Type of publication	Article (peer-reviewed)
Link to publisher's version	10.1128/aem.01801-17
Rights	Open access - http://creativecommons.org/licenses/by/4.0/© 2017, American Society for Microbiology.
Download date	2026-11-03 22:10:00
Item downloaded from	https://hdl.handle.net/10468/7134

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AEM Accepted Manuscript Posted Online 13 October 2017
Appl. Environ. Microbiol. doi:10.1128/AEM.01801-17
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Purification, characterization and heterologous production of plantaricyclin A, a novel circular bacteriocin produced by *Lactobacillus plantarum* NI326

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Running title: Plantaricyclin A, a novel circular bacteriocin from *Lb. plantarum*

Key words: circular bacteriocin, *Alicyclobacillus acidoterrestris*, *Lactobacillus plantarum*, immunity.

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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 a strain, *Lactobacillus plantarum* NI326, from olives with activity against a strain belonging to the beverage-spoilage bacterium *Alicyclobacillus acidoterrestris* spp. Genome sequencing of the strain enabled the identification of a gene cluster 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 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 subsequently cloned and expressed in *L. lactis* NZ9000, resulting in the production of an active 5,570 Da bacteriocin in the supernatant. PlcA is produced as a 91-amino acid precursor with a 33 amino acid leader peptide. This leader peptide is predicted to be removed, after which the N- and C-termini are joined *via* a covalent linkage to form the mature 58 amino acid circular bacteriocin PlcA. This is the first report of a characterized circular bacteriocin produced by *Lb. plantarum* and the inhibition displayed against *A. acidoterrestris* sp1 highlights the potential use of this bacteriocin as a preservative in food and beverages.

IMPORTANCE

In this work we describe the purification and characterization of a new antimicrobial peptide, termed Plantaricyclin A (PlcA), produced by a *Lactobacillus plantarum* strain isolated from olives. This peptide has a circular structure, and all the genes involved in its production, circularization and secretion have been identified. PlcA shows antimicrobial activity against

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42 different strains, including *Alicyclobacillus acidoterrestris*, a common beverage spoilage
43 bacteria causing important economic losses in the beverage industry every year. PlcA is the
44 first circular antimicrobial peptide described from *Lactobacillus plantarum*.
45

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INTRODUCTION

Bacteriocins are ribosomally synthesized antimicrobial peptides produced by bacteria to inhibit the growth of other, often closely related, strains. 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 non-toxic food preservatives (1, 2). Some of these peptides have demonstrated greater efficacy than conventional antibiotics against numerous pathogenic and drug-resistant bacteria, while displaying no toxicity toward eukaryotic cells (3). For this reason, bacteriocins may also be useful in human and veterinary applications as a powerful weapon in the ongoing battle against antibiotic resistance, and for the treatment of local and systemic bacterial infections (3-5).

A recent classification of bacteriocins of LAB established three main groups of these peptides (6). Class I and class II are represented by heat-stable bacteriocins (<10 kDa), while class III includes the thermo-labile bacteriocins (>10 kDa). Class I encompasses bacteriocins that undergo enzymatic modification during biosynthesis, and this class is further subdivided into six subclasses: lanthiopeptides, circular bacteriocins, sactibiotics, linear azol(in)e-containing peptides (LAPs), glycocins and lasso peptides. Class II bacteriocins include unmodified bacteriocins, and this group is subdivided into four subclasses: pediocin-like, two-peptide, leaderless and non-pediocin-like single-peptide bacteriocins. Class III includes (heat-sensitive) unmodified bacteriocins larger than 10 kDa with a bacteriolytic or non-lytic mechanism of action. This group can be further subdivided into two classes: the bacteriolysins and the non-lytic bacteriocins.

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70 Class Ib or circular bacteriocins constitute a unique family of active proteins in which the N-
71 and C-terminal ends are covalently linked to form a circular backbone. This additional bond is
72 thought to enhance the thermodynamic stability and structural integrity of the peptide and
73 consequently improve its biological activity (7-9). To date, only a small number of circular
74 bacteriocins have been described. These can be subdivided in two major groups according to
75 their physicochemical characteristics and level of sequence identity (9). Subgroup I
76 encompasses circular bacteriocins with a high content of positively charged amino acids and a
77 high isoelectric point (pI of ~10). This includes the most studied circular bacteriocin, enterocin
78 AS-48 (10), together with other bacteriocins such as carnocyclin A (11), circularin A (12),
79 lactocyclin Q (13), and garvicin ML (14). Subgroup II circular bacteriocins include
80 bacteriocins with a smaller number of positively charged amino acid residues and a medium to
81 low isoelectric point (pI between ~ 4 and 7). Currently this group comprises just three
82 members, gassericin A (15), butyrivibriocin AR10 (16) and acidocin B (17). However there is
83 an absence of consensus regarding the classification of circular bacteriocins, as some authors
84 consider that they should be grouped as Class II bacteriocins, instead of Class I (1).
85
86 In this study we screened 50 colonies, isolated from olives, for their potential to inhibit growth
87 of the beverage-spoilage strain *Alicyclobacillus acidoterrestris* sp1. We report the purification
88 and genetic characterization of a novel circular gassericin A-like bacteriocin, termed
89 plantaricyclin A produced by *Lactobacillus plantarum* NI326, with antimicrobial activity
90 against various microorganisms including *A. acidoterrestris* sp1.
91
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MATERIALS AND METHODS

Cultures and growth conditions

The strains used in this study are summarized in Table 1. All *Lactobacillus*, *Pediococcus* and *Leuconostoc* strains were grown in MRS (Oxoid, Hampshire, U.K.) at 30 °C, *A. acidoterrestris* sp1 was grown in 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 typhimurium* and *Klebsiella pneumoniae*), 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 reqired. All microorganisms were grown under aerobic conditions. All strains were stored at -80 °C in their respective media with 20 % glycerol until required.

Isolation of LAB strains from olives

Over 50 isolates were isolated from olives as previously described (18). Briefly, 5 g olives were homogenised with 45 ml Ringers solution using a stomacher at 300 bpm for 1 min (Stomacher circular 400, Seward, UK). Homogenate was serially diluted in Ringers 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.

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118

119 **Isolation of anti-*A. acidoterrestris* sp1 bacteriocin-producing LAB**

120 LABs exerting antimicrobial activity were identified using the spot-on-lawn method (18).
121 Briefly, 5 µl aliquots of LAB cultures were spotted onto MRS agar plates and grown at 30 °C
122 anaerobically for 48 h. Plates were then overlaid with 5 mL of MRS soft agar (MRS broth
123 supplemented with 0.8 % bacteriological agar) seeded with 10^5 – 10^6 CFU/mL of an overnight
124 culture of *L. lactis* HP. Plates were incubated at 30 °C for 48 h after which zones of inhibition
125 surrounding the LAB colony were measured.

126 The LAB isolate showing inhibition against *L. lactis* HP was further cultured in 10 ml MRS
127 broth and grown at 30 °C overnight. Cell-free culture supernatant (CFS) was obtained by
128 centrifugation of the culture at 12,000 g, 4 °C for 10 min and filtered through 0.2 µm pore-size
129 filters (Whatman Int. Ltd., Maidstone, UK). The activity of the CFS against *A. acidoterrestris*
130 sp1 was analysed using an agar diffusion test (ADT) (19). Briefly, 100 µl aliquots of CFS were
131 placed in wells (6-mm diameter) bored in cooled Alicyclobacillus agar (Pronadisa) plates (30
132 ml) previously seeded (10^5 CFU/ml) with *A. acidoterrestris* sp1. Plates were incubated at 50
133 °C to allow growth of the target organism and checked for zones of inhibition after 24-48 h.

134

135 **Identification of LAB isolates**

136 Individual colonies were used as templates for PCR. The primers Luc-F (5' CTT GTT ACG
137 ACT TCA CCC 3') and Luc-R (5' TGC CTA ATA CAT GCA AGT 3') (Eurofins MWG,
138 Ebersberg, Germany) were used to amplify a variable region of the 16S rRNA gene (20). The
139 following conditions were used for the PCR reactions: 95 °C for 60 s, 53 °C for 60 s, and 72
140 °C for 95 s, for 30 cycles. The DNA from individually purified amplicons was subjected to
141 Sanger sequencing (Eurofins MWG) and the corresponding species identity was obtained by
142 comparative sequence analysis (BLASTN) against available sequence data in the National

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143 Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/
144 BLAST).
145
146 ***Lactobacillus. plantarum*** **NI326 genome sequencing, genome annotation and bacteriocin**
147 **screening**
148 The genome of *Lb. plantarum* NI326 was sequenced using a combined Roche GS-FLX
149 Titanium and Illumina HiSeq 2000 approach (GATC Biotech, Konstanz, Germany), to a final
150 coverage of ~490-fold. Sequences obtained were first quality checked using IlluQC.pl from
151 the NGS QC Toolkit (v2.3) (http://www.nipgr.res.in/ngsqctoolkit.html) (21) and assembled
152 with AbySS (v1.9.0) (22).
153 Following sequence assembly, the generated contigs were employed to perform Open Reading
154 Frame (ORF) prediction with Prodigal v2.5 prediction software (http://prodigal.ornl.gov),
155 supported by BLASTX v2.2.26 alignments (23). ORFs were automatically annotated using
156 BLASTP v2.2.26 (23) analysis against the non-redundant protein databases curated by the
157 NCBI Database. Following automatic annotation, ORFs were manually curated using Artemis
158 v16 genome browser and annotation tool (http://www.sanger.ac.uk/science/tools/artemis). The
159 software tool was used to inspect and validate ORF results, to adjust start codons where
160 necessary, and to aid in the identification of pseudogenes. The resulting ORF annotations were
161 further refined, where required, using alternative databases; Pfam (24) and Uniprot/EMBL
162 (http://www.uniprot.org/). Transfer tRNA was predicted using tRNA-scan-SE v1.4 (25). The
163 whole genome was analysed with the web-based bacteriocin genome mining tool BAGEL3
164 (http://bagel.molgenrug.nl/) (26) to search for known and/or potential novel bacteriocins.
165
166 **Accession numbers**

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167 The GenBank accession number provided for the nucleotide sequence reported in this study is
168 NDXC00000000.
169
170 **Molecular cloning of *plc* gene cluster, *plcD* and *plcI* into pNZ8048 and transformation in**
171 ***L. lactis* NZ9000**
172 The primers, PCR fragments and plasmids used in this study are listed in Table 2. All primers
173 were ordered from Eurofins MWG. Plasmid derivatives were constructed as follows: primers
174 Plc-F/Plc-R were used for PCR-amplification of a 3,172-bp fragment from total genomic DNA
175 of *Lb. plantarum* NI326, which encompassed the entire *plc* gene cluster including its
176 promoter(s). Using this *plc* gene cluster fragment as template and the primer pairs NcoI-
177 Plc/XbaI-Plc, NcoI-PlcD/XbaI-PlcD, NcoI-PlcI/XbaI-PlcI and NcoI-PlcD/XbaI-PlcI,
178 fragments encompassing *plcADITEB*, *plcD*, *plcI* and *plcDI*, respectively, were amplified
179 (Table 3). Such fragments were digested with *Nco*I and *Xba*I and ligated into pNZ8048,
180 digested with the same enzymes. The ligation mixtures were used to transform *L. lactis*
181 NZ9000 competent cells as previously described (27). The plasmid derivatives pNZPlc,
182 pNZPlcD, pNZPlcI and pNZPlcDI, were checked by colony-PCR and sequencing of the
183 inserts using primers PNZ-F/PNZ-R.
184
185 **Purification and MALDI TOF mass spectrometry analysis of PlcA**
186 PlcA was purified from *Lb. plantarum* NI326 and *L. lactis* NZ9000 transformed with pNZPlc,
187 as described previously (28) with modifications. Briefly, a 1 L CFS of *Lb. plantarum* NI326
188 was obtained as previously described. Recombinant *L. lactis* NZ9000 – pNZPlc was induced
189 for the production of PlcA at an optical density at 600 nm (OD600) of 0.5, using nisin A
190 (Nisaplin, Dupont, USA) at a final concentration of 10 ng/ml. The induced culture was grown
191 at 32 °C for 3 h. CFS was obtained by centrifugation of the culture at 12,000 × *g* at 4 °C for 10

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192 min. Activity of the CFS from either strain against *A. acidoterrestris* sp1 was confirmed on an
193 ADT as previously described. CFS was applied to a 10g (60 ml) Varian C-18 Bond Elution
194 Column (Varian, Harbor City, CA) pre-equilibrated with methanol and water. The column was
195 washed with 20 % ethanol and the inhibitory activity was eluted in 100 mls of 70 % 2-
196 propanol 0.1 % TFA. 15 ml aliquots were concentrated to 2 ml through the removal of 2-
197 propanol by rotary evaporation (Buchi). Samples were then applied to a semi preparative
198 Vydac C4 Mass Spec (10 x 250 mm, 300Å, 5µ) RP-HPLC column (Grace, Columbia, USA)
199 running an acetonitrile and propan-2-ol gradient described as follows: 5-55 % buffer B and 0-5
200 % buffer C over 25 minutes followed by and 55-19 % buffer B and 5-65 % buffer C over 60
201 minutes, 19-5 % buffer B and 65-95 % buffer C over 5 minutes where buffer A is Milli Q
202 water containing 0.1 % TFA, buffer B is 90 % acetonitrile 0.1 % TFA and buffer C is 90 %
203 propan-2-ol 0.1 % TFA. Eluent was monitored at 214 nm and fractions were collected at 1
204 minute intervals. Fractions were assayed on *Lactobacillus bulgaricus* indicator plates and
205 active fractions assayed for the antimicrobial mass of interest using MALDI TOF mass
206 spectrometry (MALDI TOF MS). MALDI TOF MS was performed with an Axima TOF^2
207 MALDI TOF mass spectrometer (Shimadzu Biotech, Manchester, UK) as described by Field
208 et al (28).
209
210 **Analysis of Immunity against PlcA**
211 The immunity of wild type *L. lactis* NZ9000 and recombinant strains *L. lactis* NZ9000 –
212 pNZPlcD, *L. lactis* NZ9000 – pNZPlcI and *L. lactis* NZ9000 – pNZPlcDI was tested against
213 CFS from *Lb. plantarum* NI326 on an ADT assay as above described. The indicator strains
214 were seeded in GM17 – 0.8 % agar with and without 10 ng/ml Nisin A. The area of zones of
215 inhibition was measured after 24 hours growth at 30 °C. The absence of a zone indicates that
216 the strain is immune to PlcA.

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Sensitivity of PlcA to heat, pH and proteolytic enzymes

Aliquots of PlcA-containing fraction obtained following Reversed Phase HPLC were subjected to the following treatments: (i) 20-fold (v/v) dilution with 30 % 2-propanol containing 0.1 % TFA and heating at 80 ºC and 100 ºC for 30 min and at 121 ºC for 15 min to determine the stability of PlcA to heat; (ii) 20-fold (v/v) dilution in 10 mM Tris buffer followed by 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 in (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.

Antimicrobial spectrum of PlcA

Aliquots of PlcA were used to test its antimicrobial activity against various indicators (Table 1) using an ADT assay as described above.

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RESULTS AND DISCUSSION

Alicyclobacillus acidoterrestris is considered to be one of the species with the highest food spoilage impact worldwide (29). They are thermo-acidophilic spore-forming bacteria 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 odour 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 as strategies to inhibit growth of *A. acidoterrestris* in juices (30, 31).

Isolation and identification of *Lactobacillus plantarum* NI326.

In this study we screened a number of isolates of LAB from olives with the aim of selecting those showing antimicrobial activity against *A. acidoterrestris* sp1. 50 potential LAB isolates were obtained from the olive homogenate plated on MRS plates. Single colonies were streaked onto fresh MRS plates and overlaid with *L. lactis* HP. Only one out of the 50 tested colonies exhibited a zone of inhibition against the indicator strain. A CFS of this strain produced an inhibitory zone against *A. acidoterrestris* sp1 on an ADT, confirming that this isolate produces an extracellular antimicrobial compound against *A. acidoterrestris* sp1. This colony was identified as *Lb. plantarum* by 16S rDNA sequencing and designated *Lb. plantarum* NI326. No zone of inhibition was apparent when the CFS was first treated with proteinase K confirming the proteinaceous nature of the antimicrobial compound (data not shown).

Genome sequence analysis and annotation bacteriocin encoding gene cluster of *Lb. plantarum* NI326.

To find potential bacteriocin-encoding gene clusters, the entire genome of *Lb. plantarum* NI326 was sequenced generating 84 contigs following sequence assembly. *In silico* analysis of

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262 the 84 contigs with BAGEL3 detected a potential bacteriocin gene cluster predicted to encod a
263 peptide with a 43-AA putative conserved domain corresponding to the class Ib-subgroup II
264 gassericin A-like circular bacteriocins. This putative peptide, designated here as plantaricyclin
265 A (PlcA), exhibits 67 % similarity to the circular bacteriocin gassericin A. An alignment of
266 this peptide with the other members of the gassericin A-like circular bacteriocin group:
267 gassericin A (GaaA), acidocin B (AciB) and butyrivibriocin AR10 (BviA), revealed a high
268 degree of similarity with PlcA facilitated the prediction of the potential cleavage site of the
269 signal peptide from the mature peptide to be between amino acids N33 and I34 (Figure 1).
270 Both GaaA and AciB are synthesized as 91 AA pre-peptides with 33 AA leader peptides that
271 are cleaved off, followed by a covalent linkage between the N- and C-terminus, to form the
272 mature 58 amino acid circular bacteriocin. In previous studies, sequence alignments between
273 characterized and hypothetical subgroup II circular bacteriocins has revealed the presence of a
274 fully conserved asparaginyl cleavage site (17), which is also present in PlcA.
275
276 The function of these leader peptides and mechanism through which peptide circularization
277 occurs is still unclear. One of the biggest challenges in the field of circular proteins is finding
278 out how their ends are stitched together from their linear precursors (32). Identification of the
279 mechanism involved has to potential to facilitate the creation of new, highly stable
280 antimicrobial agents for use in food, veterinary and medical applications (11). PlcA has a
281 predicted mass of 5,588 Da and represents a new bacteriocin within the Class Ib Subgroup II
282 and the first (predicted) circular bacteriocin isolated from *Lb. plantarum*.
283
284 Analysis of the PlcA gene cluster revealed the presence of seven ORFs downstream of the
285 PlcA-encoding gene (*plcA*), with sequence and organisational similarity to those found in the
286 gene clusters responsible for GaaA and AciB production (Figure 2). Accordingly, *plcA* is

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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 (33). ORF *plcI* is immediately downstream of *plcD*, and encodes a 54 AA protein with a hypothetical function as an immunity protein. Kawai et al (34) showed that heterologous expression of GaaI in *Lactococcus lactis* confers a 7-fold higher resistance to gassericin A compared to a control strain.

The next two genes of the cluster (*plcE* and *plcT*) encode proteins of 227 AA and 214 AA, respectively. Both have conserved ATP-binding domains linked to proteins of the ABC transporter family and based on homology to their equivalents from GaaA and AciB clusters, they are most likely involved in the secretion of PlcA. The downstream *plcB* and *plcC* genes are located in positions that are different from their homologs in the clusters for GaaA and AciB production (Figure 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 must play an important role (8).

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 exhibited antimicrobial activity against *A. acidoterrestris* sp1 similar to that from the wildtype *Lb. plantarum* NI326 (Figure 3a). The production of PlcA by *L. lactis* confirms that the cluster contains all the information necessary for the correct production, modification and secretion of PlcA. Based on these results and the similarity of the *plc* cluster

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312 to those from GaaA and AciB, we can hypothesize that the biosynthetic machinery for all
313 members of this bacteriocin subgroup is similar.
314
315 **Analysis of immunity to PlcA**
316 In order to determine if *plcD* and/or *plcI* code immunity proteins for PlcA, the genes were
317 cloned individually or together in the NisA-inducible vector pNZ8048 and transformed into *L.*
318 *lactis* NZ9000. The recombinant strain *L. lactis* NZ9000 – pNZPlcDI induced with nisin A
319 displayed full resistance to PlcA while strains *L. lactis* NZ9000 – pNZPlcD and *L. lactis*
320 NZ9000 – pNZPlcI induced with nisA showed 86 % and 62 % sensitivity against PlcA,
321 respectively, in comparison to the activity of the bacteriocin against the control strain *L. lactis*
322 NZ9000 – pNZ8048 (Figure 3b). Therefore, although both proteins individually appeared to
323 confer partial immunity to *L. lactis* NZ9000 against the antimicrobial activity of PlcA, the
324 recombinant strain was fully protected against the action of PlcA when both proteins were
325 being produced concomitantly. Similar results have been observed with other circular
326 bacteriocins such as carnocyclin A, where the production of the immunity protein (CclI) was
327 not enough to confer full protection to the producer and only when CclD and CclI were co-
328 produced did the strain show full immunity (35).
329
330 **Purification and MALDI TOF analyses of the antimicrobial activity of *Lb. plantarum***
331 **NI326**
332 The antimicrobial peptide produced by *Lb. plantarum* NI326 and *L. lactis* pNZPlc CFS was
333 purified by Reversed Phase-HPLC and the molecular mass analyzed by MALDI TOF MS. In
334 both cases a single mass of 5,572 Da was detected in the active fractions (Figure 4). The 18 Da
335 difference between the molecular mass of PlcA and its theoretical mass calculated from the

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AA sequence corresponds to the loss of a molecule of water that occurs during circularization of the peptide as reported for other circular bacteriocins (17, 36).

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 ºC to 100 ºC 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 mainly due 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 allow the formation of a central pore, resembling the structure of the saposins. The surface of acidocin B and gassericin A is 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 (17).

Antimicrobial spectrum of plantaricyclin A.

Aliquots of the HPLC purified fractions of PlcA were evaluated for their antimicrobial activity and inhibitory spectrum against different indicator microorganisms. Of these only *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

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361 1). In comparison with other circular bacteriocins, PlcA possesses a narrow spectrum of
362 activity. The low yields obtained during the purification of PlcA may explain the lack of
363 activity against some of the indicators used.
364
365 In addition to the spectra of inhibition, we observed some other differences between PlcA and
366 the other members of subgroup II, such as a higher isoelectric point (8.6) and a net charge of
367 +1. In fact some authors use the pI values and net charges to differentiate between circular
368 bacteriocins of subgroup I (pI~10 and positively charged) from circular bacteriocins of
369 subgroup II (pI 4 to 7 and uncharged or slightly negative) (9). According to this classification
370 system PlcA should be placed in an intermediate position between subgroups I and II.
371 However, we strongly believe that this peptide should be classified within subgroup II and
372 propose to modify the classification criteria and broaden the pI range for this subgroup to be
373 between 4 to ~9.
374
375 The peptide plantaricyclin A is the first circular bacteriocin isolated and characterized from a
376 *Lb. plantarum* strain. The high level of antimicrobial activity observed against the food and
377 beverage spoilage microorganism *Alicyclobacillus acidoterrestris* is of great interest as this
378 strain represents a significant problem for the food industry. The use of bacteriocins, such as
379 nisin A and enterocin AS-48, as preservatives in low pH beverages and juices has shown some
380 promising results to control the growth of *A. acidoterrestris* (37). The circular nature of PlcA
381 makes it especially interesting for industrial applications as this peptide could survive and
382 retain most of the activity under changing conditions (temperature and pH, for example)
383 during food/beverage manufacture. Moreover, the narrow spectrum of activity from PlcA can
384 be considered as an advantage specially in fermented beverages. In comparison to other broad
385 spectrum bacteriocins such as nisin A or enterocin AS-48, PlcA could be used to specifically

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target *A. acidoterrestris* spp while having little or no effect against other desirable microorganisms present in the beverage.

ACKNOWLEDGEMENTS

This work was supported by a grant from Enterprise Ireland – Innovation Partnership Programme IP/2013/0254. DvS is a member of the APC Microbiome Institute funded by Science Foundation Ireland (SFI), through the Irish Government's National Development Plan (Grant number SFI/12/RC/2273). JM is the recipient of a Starting Investigator Research Grant funded by SFI (Ref. No. 15/SIRG/3430).

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545 Table 1. Strains used in this study, sources, and activity of PlcA (+: zone of inhibition
546 observed; - : no zone of inhibition observed).
547

Strain	Source	Activity
Alicyclobacillus acidoterrestris sp1	Coca Cola	+
Lactococcus lactis HP	UCC	+
Lactococcus lactis KH	UCC	+
Lactococcus lactis MG1363	UCC	+
Lactococcus lactis RT28	UCC	+
Lactococcus lactis NZ9000	UCC	+
Lactobacillus bulgaricus UCC	UCC	+
Lactobacillus plantarum -PARA	UCC	-
Lactobacillus plantarum WCFSI	UCC	-
Lactobacillus brevis MB124	UCC	-
Lactobacillus brevis SAC12	UCC	-
Lactobacillus brevis L102	UCC	-
Lactobacillus brevis L94	UCC	-
Pediococcus claussenii H5	UCC	-
Pediococcus inopinatus 1011	UCC	+
Enterococcus faecium DPC1146	UCC	-
Listeria innocua UCC	UCC	-
Listeria monocytogenes EgDe	UCC	-
Listeria monocytogenes 33077	UCC	-
Escherichia coli EC10B	UCC	-
Staphylococcus aureus DPC5243	UCC	-
Streptococcus uberis ATCC700407	UCC	-
Streptococcus dysgalactiae GrpC	UCC	-
Salmonella typhimurium UTC1lux	UCC	-
Klebsiella pneumoniae UCC	UCC	-
Bacillus cereus DPC6087	UCC	-

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Table 2. Putative proteins derived from the *plca* operon

ORF	Length (amino acids)	Amino acids indentity (%) relative to gassericin A gene cluster homologs	Hypothetical function	Genbank accession no.
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	

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Table 3. Primers, PCR products and plasmids used in this study.

Primer	Nucleotide sequence (5' - 3')[a]	PCR fragment
Plc-F	AACGCAAATGTTCCACACGG	Plc-Clust
Plc-R	GGATTGGACTAGTAGCTCTAGGGT	Plc-Clust
NcoI-Plc	CACTCACCATGGGTTAATGCTTTCAGCATATCGTAGTAAAT	PlcADITEB
XbaI-Plc	ATCTATCTAGACTATAAAAAAATCAAGCTATATATAGG	PlcADITEB
NcoI-PlcD	CACTCACCATGGTGAATAAACCGCGGAGTAATATC	PlcD / PlcDI
XbaI-PlcD	ATCTATCTAGATTAATCTCCTAACAACCATAAGGC	PlcD
NcoI-PlcI	CACTCACCATGGTTGTTAGGAGATTAATTATGAAGAATTTAG	PlcI
XbaI-PlcI	ATCTATCTAGATTAATCTGTATGCCGTTTAATTAGCTGA	PlcI / PlcDI
pNZ-F	TGTCGATAACGCGAGCATAA	
pNZ-R	CAAAGCAACACGTGCTGTAA	

PCR fragment	Description
Plc-Clust	3,172-bp fragment external to Plc cluster
PlcADITEB	2,908-bp *Nco*I/*Xba*I fragment containing genes *plcA*, *plcD*, *plcI*, *plcT*, *plcE* and *plcB*
PlcD	495-bp *Nco*I/*Xba*I fragment containing gene *plcD*
PlcI	204-bp *Nco*I/*Xba*I fragment containing gene *plcI*
PlcDI	662-bp *Nco*I/*Xba*I fragment containing genes *plcD* and *plcI*

Plasmid	Description
pNZ8048	Cm^r; inducible expression vector carrying the nisA promoter (38)
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 in the primers.

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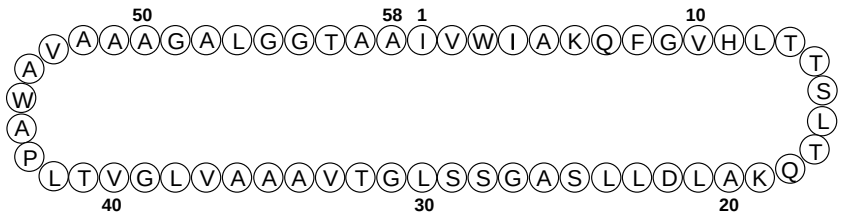
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Figure 1. A) Sequence alignment of all the members of subgroup II circular bacteriocins with plantaricyclin A, using MUSCLE (39). Conserved. Conservative and semiconservative substitutions are indicated by asterisks, colons, and semicolons, respectively. Bold letters determine the leader sequence. B) Schematic plantaricyclin A mature peptide.

A)

```
Gassericin A          MVTKYGRNLGLNKVELFAIWAVLVVALLLTTAN IYWIADQFGIHLATGTARKLLDAMASGASLGTAFAAILGVTLPAWALAAAGALGATAA 91
Acidocin B            MVTKYGRNLGLSKVELFAIWAVLVVALLLATAN IYWIADQFGIHLATGTARKLLDAVASGASLGTAFAAILGVTLPAWALAAAGALGATAA 91
Butyrivibriocin AR10            MSKKQIMSNCISIALLIALIPN IYFIADKMGIQLAPAWYQDIVNWVSAGGTLTTGFAIIVGVTVPAWIAEAAAAFGIASA 80
Plantaricyclin A      MLSAYRSKLGLNKFEVTVLMIISLFILLFATVN IVWIAKQFGVHLTTSLTQKALDLLSAGSSLGTVAAAVLGVTLPAWAVAAAGALGGTAA 91
                                :.* ::     : :  *:   * * :**.::*::*: .  :. :: :::*.:* *  * ::***:***   **.*:* ::*
```

B)



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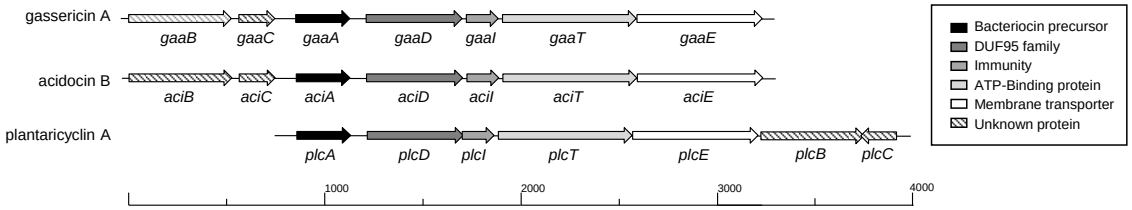
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Figure 2. Schematic representation of the gene clusters involved in the production of the circular bacteriocins gassericin A (34), acidocin B (17) and plantaricyclin A. The known or putative biochemical function or properties are denoted by color, as indicated in the key.



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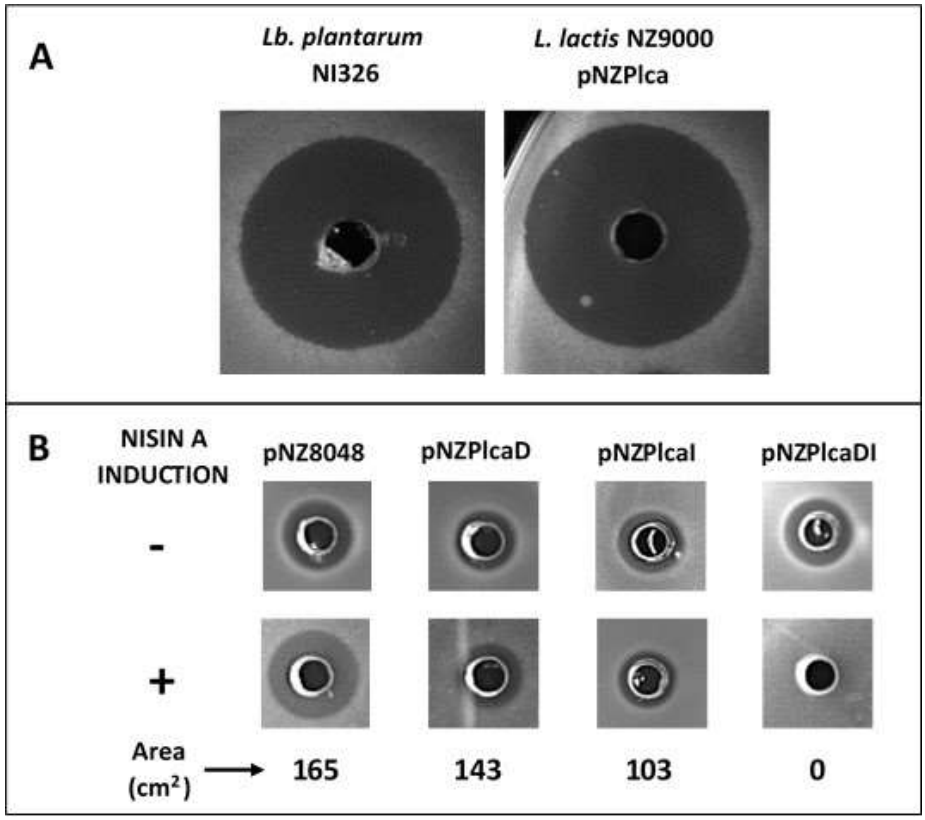


Figure 3. A) Antimicrobial activity of the CFS of *Lb. plantarum* NI326 and nisin A-induced *L. lactis* NZ9000 pNZPlc against *A. acidoterrestris* sp1. B) Antimicrobial activity of the CFS of *Lb. plantarum* NI326 against cultures of *L. lactis* NZ9000 pNZ8048, *L. lactis* NZ9000 pNZPlcD, *L. lactis*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

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Figure 4. MALDI TOF Mass spectrometry analysis of the purified plantaricyclin A produced by A) *L. lactis* pNZPlca; and B) *Lb. plantarum* NI326

