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Cloning and functional expression of a food-grade circular bacteriocin, plantacyclin B21AG, in probiotic *Lactobacillus plantarum* WCFS1

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Abstract

There is an increasing consumer demand for minimally processed, preservative free and microbiologically safe food. These factors, combined with risks of antibiotic resistance, have led to interest in bacteriocins produced by lactic acid bacteria (LAB) as natural food preservatives and as potential protein therapeutics. We previously reported the discovery of plantacyclin B21AG, a circular bacteriocin produced by *Lactobacillus plantarum* B21. Here, we describe the cloning and functional expression of the bacteriocin gene cluster in the probiotic *Lactobacillus plantarum* WCFS1. Genome sequencing demonstrated that the bacteriocin is encoded on a 20 kb native plasmid, designated as pB21AG01. Seven open reading frames (ORFs) putatively involved in bacteriocin production, secretion and immunity were cloned into an *E. coli*/*Lactobacillus* shuttle vector, pTRKH2. The resulting plasmid, pCycB21, was transformed into *L. plantarum* WCFS1. The cell free supernatants (CFS) of both B21 and WCFS1 (pCycB21) showed an antimicrobial activity of 800 AU/mL when tested against WCFS1 (pTRKH2) as the indicator strain, showing that functional expression of plantacyclin B21AG had been achieved. Real-time PCR analysis revealed that the relative copy number of pB21AG01 was 7.60 ± 0.79 in *L. plantarum* B21 whilst pCycB21 and pTRKH2 was 0.51 ± 0.05 and 25.19 ± 2.68 copies respectively in WCFS1. This indicates that the bacteriocin gene cluster is located on a highly stable low copy number plasmid pB21AG01 in *L. plantarum* B21. Inclusion of the native promoter for the bacteriocin operon from pB21AG01 results in similar killing activity being observed in both the wild type and recombinant hosts despite the lower copy number of pCycB21.

Introduction

Bacteriocins are ribosomally synthesised, extracellularly released peptides or peptide complexes that possess antibacterial activity against species usually closely related to the producer strains or a wider range of microorganisms [1, 2]. Interestingly, the bacteriocins produced by

Gram-positive bacteria seem to exhibit broader spectrum activity compared to those of Gram-negative bacteria [3]. Amongst the Gram-positive bacteria, bacteriocins produced by the food-grade lactic acid bacteria (LAB) have attracted considerable interest because they are generally regarded as safe (GRAS). Being proteins, they can be easily degraded by proteases in the mammalian gastrointestinal tract, making them safe for human consumption and minimizing the risk of developing resistant bacteria [4, 5]. They have been widely used as natural food preservatives for controlling food-borne and food-spoilage bacteria without affecting food sensory qualities. They also have huge potential in veterinary applications and as next-generation antibiotics against multi-drug resistant (MDR) pathogens [5–7]. One of the advantages of bacteriocins over conventional antibiotics is that they are directly gene encoded, making bioengineering and evolutionary approaches to tailor and enhance their action against specific pathogens entirely feasible [5, 8].

The classification of bacteriocins produced by Gram-positive bacteria has been constantly revised due to the extensive research performed over the last two decade [9–11]. Here we use the classification proposed by Acedo et al. [12]. Class I contains modified peptides including lantibiotics, lipolanthines, linear azol(in)e-containing bacteriocins, thiopeptides, bottromycins, sactibiotics, lasso peptides, glycocins and circular bacteriocins. Class II are unmodified peptides such as YGNG-motif containing bacteriocins, two-peptide bacteriocins, leaderless bacteriocins and other linear bacteriocins. Class III are large heat labile bacteriocins such as bacteriolysins, non-lytic large bacteriocins and tailocins. Of these, the class I circular bacteriocins have gained considerable attention as they generally exhibit broad antimicrobial activity. They are synthesised as linear pre-peptides where the leader peptides of variable sizes (2–48 amino acids) are cleaved off during maturation, forming 58–70 amino acid peptides which are covalently linked by a largely unknown cyclisation mechanism [13, 14]. The circular structures appear to enhance their pH and thermal stability as well as protease resistance. These properties make them a preferred candidate for potential industrial applications compared to the other classes of bacteriocins [13, 15]. Development of a suitable native expression system(s) would allow both structure-function and bioengineering studies to be carried out.

Among the LAB, bacteriocins produced by *Lactobacillus*, in particular *Lactobacillus plantarum*, have been widely studied for several reasons. *L. plantarum* is a versatile species that is widely found in a variety of sources, including meat, dairy, fish, fruit and vegetables [16]. It is also one of the natural inhabitants of the human gastrointestinal tract (GIT) where its ability to survive passage through the GIT makes it an attractive vector for vaccine delivery [17, 18]. The availability of the complete genome sequence of *L. plantarum* WCFS1 and genome mining tools have facilitated the characterisation of the genetic organisation of the classical plantaricin (*pln*) loci from this species [19]. Hitherto, several other class II linear two-peptide bacteriocins produced by *L. plantarum* strains have been described. For example, plantaricin C19 produced by *L. plantarum* C19, isolated from fermented cucumber, and plantaricin NA produced by *L. plantarum*, isolated from 'ugba', an African fermented oil-bean seed, which showed strong antimicrobial activity against the food-borne pathogen, *Listeria monocytogenes* [20, 21]. Bacteriocin AMA-K produced by *L. plantarum* AMA-K, isolated from fermented milk exhibited strong adsorption to cells of *L. monocytogenes*, *L. ivanovii* subsp. *ivanovii* and *L. innocua* [22]. Plantaricin ST8KF produced by *L. plantarum* ST8KF, isolated from kefir, demonstrated antimicrobial activity against *L. casei*, *L. salivarius*, *L. curvatus*, *Enterococcus mundtii* and *L. innocua* [23, 24]. In contrast, only one circular bacteriocin, plantaricyclin A produced by *L. plantarum* NI326, has been reported to date. Similarly, this circular bacteriocin is active against beverage-spoilage bacterium *Alicyclobacillus acidoterrestris* [25]. These antimicrobial peptides appear to have great potential in food preservation, particularly in controlling food-borne

pathogens. Discovery of new circular bacteriocins with enhanced pathogen killing is preferred over linear bacteriocin peptides due to their superior stability and resistance to proteases [26].

In recent years, research on bacteriocins has progressed from producing the inhibitory compounds in native systems to heterologous production in diverse producer organisms which have the potential to be employed as starters, protectors and/or probiotics [27]. Several strategies for heterologous expression of bacteriocins have been investigated either for over-production of the bacteriocin or structure-function studies [7, 27–29]. We previously reported the discovery of plantacyclin B21AG, a food-grade circular bacteriocin produced by *Lactobacillus plantarum* B21 [30, 31]. It was shown to be active against food-borne pathogens including *Clostridium perfringens* and *Listeria monocytogenes*; food spoilage bacteria such as *L. arabinosus*; as well as other LAB including *L. plantarum*, *L. brevis* and *Lactococcus lactis* [30–32]. This study aimed to transfer the ability to produce the broad antimicrobial activity of plantacyclin B21AG to a standard probiotic strain, *L. plantarum* WCFS1 [33]. We have demonstrated that the bacteriocin gene cluster can be recombinantly expressed in *L. plantarum* WCFS1 at a level comparable to the native producer *L. plantarum* B21. The mobilization of the plantacyclin B21AG operon into the probiotic, *L. plantarum* WCFS1, enhances the antimicrobial activity of the strain, potentially making it more useful for use in the food industry and for clinical applications. Having demonstrated this in principle, non-GMO approaches such as transfer of the natural plasmid to other probiotic strains can now be attempted with suitable selection strategies.

Materials and methods

Bacterial strains, plasmids and culture conditions

Bacterial strains and plasmids used in this study are listed in Table 1. All *Lactobacillus* strains were cultured statically in deMan, Rogosa and Sharpe (MRS) broth (Becton, Dickinson and Company, USA) at 37˚C under aerobic conditions. *Escherichia coli* strains were grown in Luria-Bertani (LB) medium (Becton, Dickinson and Company, USA) at 37˚C with continuous agitation at 250 rpm. For selection, media were supplemented with 100 μg/mL of ampicillin and/or 150 μg/mL of erythromycin for *E. coli* and 15 μg/mL of erythromycin for *Lactobacillus*.

Sequence determination and genetic analysis of pB21AG01

The full genome of *L. plantarum* B21, including the 20.4 kb plasmid, pB21AG01, was sequenced at the Beijing Genomics Institute (BGI) using the Illumina HiSeq 2000 platform (Illumina, USA) and assembled with SOAPdenovo software [36]. The plasmid was annotated using RAST [37]. The resulting open reading frames (ORFs) were confirmed using blastp against the NCBI non-redundant protein database [38]. The obtained putative protein sequences were searched for conserved domains using the NCBI Conserved Domain Database (CDD) site [39] and also examined for transmembrane domains using the TMHMM transmembrane prediction algorithm at http://www.cbs.dtu.dk [40].

DNA manipulations, plasmid constructions and *E. coli* transformations

Total gDNA from *E. coli* and *Lactobacillus* was isolated using the GeneElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich, USA) as described in the user manual. Plasmids from *E. coli* were extracted using the ISOLATE II Plasmid Mini kit (Bioline, Australia) according to manufacturer's instruction. Plasmids from *Lactobacillus* were prepared using QIAGEN® Plasmid Midi Kit (Qiagen, Germany) following supplier's direction with a few modifications to cell wall lysis. One hundred millilitres of overnight culture was harvested by centrifugation and

Table 1. Bacterial strains, plasmids and primers.

Strain/plasmid	Characteristics[a]	Reference/ Source
Strains		
Lactobacillus plantarum		
B21	Wild-type strain; plantacyclin B21AG producer	[34]
WCFS1	Wild-type strain obtained from NIZO[b]	[19]
WCFS1 (pCycB21)	*L. plantarum* type strain transformed with pCycB21; Em^r	This study
WCFS1 (pTRKH2)	Indicator strain; Bac^-; sensitive to plantacyclin B21AG	This study
Escherichia coli		
JM110 (pTRKH2)	*E. coli* strain containing pTRKH2; Amp^r Em^r	[35]
DH5α	Host strain; *fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	NEB
Plasmids		
pB21AG01	20.4 kb; native plasmid in *L. plantarum* B21; contains seven genes necessary for the production, immunity, export and processing of plantacyclin B21AG	This study
pTRKH2	6.7 kb; *E. coli*/*L. plantarum* shuttle vector; Amp^r Em^r	[35]
pCycB21	10.1 kb; contains the full plantacyclin operon cloned into the *Bam*HI and *Sac*I sites of pTRKH2; Em^r	This study
Primers		
B21AG_F	CTGCAGGGATCCGTTCAACCTCCTTTCTGAC	
B21AG_R	GGCCGGGAGCTCACAGGTTCTTAGAATACTG	

[a] Em^r and Amp^r, erythromycin and ampicillin resistant, respectively; bac^-, bacteriocin non-producing

[b] NIZO food research, The Netherlands

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washed in 20 mL STE buffer (6.7% sucrose; 50 mM Tris-HCl, pH 8.0; 1 mM EDTA) [41] to remove and neutralise acids produced during cell growth. The bacterial pellet was then resuspended in 4 mL STE buffer containing 10 mg/mL lysozyme and incubated at 37˚C for 1 hour.

For the construction of pCycB21, the full plantacyclin B21AG operon with its native promoter (a 3.4 kb fragment encompassing *orf19 –orf25*) was amplified from *L. plantarum* B21 plasmids using One*Taq*® 2X Master Mix with Standard Buffer (NEB, USA) in a T100™ Thermal Cycler (Bio-Rad, USA). The gene cluster contains seven genes required for maturation, immunity, and secretion of the circular bacteriocin outside the cells, thus obviating the need for a classical signal peptide. PCR, using the primers indicated in Table 1, was performed as follows: initial denaturation for 30 s at 94˚C; followed by 30 cycles of denaturation for 30 s at 94˚C, annealing for 30 s at 55˚C and extension for 4 min at 68˚C; and a final extension for 10 min at 68˚C. The amplified product was purified using the ISOLATE II PCR and Gel Kit (Bioline, Australia) and cloned into the *Bam*HI and *Sac*I sites of the *E. coli*/*Lactobacillus* shuttle vector, pTRKH2. The construct was transformed into *E. coli* DH5α (NEB, USA), according to the manufacturer's protocol, in order to obtain sufficient amount of plasmid DNA for subsequent transformation into LAB. The recombinant plasmid was confirmed by PCR, double restriction enzyme digestion, and DNA sequencing.

Electroporation of LAB

Electroporation of LAB was performed as described by Mason et al. [42] with a few modifications. Briefly, 8 mL of overnight LAB cultures were diluted into 40 mL of fresh, pre-warmed MRS broth containing 2% glycine. The diluted culture was incubated for 1.5 hr at 37˚C. The cells were pelleted by centrifugation at 4000 × g for 2 min at 4˚C and washed with 40 mL of

ice-cold Milli-Q water. Cells were then resuspended in 40 mL of ice-cold 50 mM EDTA and incubated on ice for 5 min. Centrifugation was repeated followed by washing of the cells in 40 mL of ice-cold 0.3 M sucrose. The cells were resuspended in 200 μL of 0.6 M sucrose. Finally, 3 μg of DNA in 50 μL of sterile Milli-Q water was added to 50 μL of freshly prepared competent cells and transferred into a pre-chilled electrocuvette with a 0.2-cm electrode gap (Cell Projects, UK). The cell suspension containing plasmid DNA was electroporated using a Gene Pulser electroporator (Bio-Rad, USA) with the following parameters: 1.5 kV, 200 Ω parallel resistance and 25 μF capacitance. The cells were transferred immediately after electroporation into 1.3 mL of pre-warmed MRS broth and incubated for 3 hrs at 37˚C. Two hundred microliters of the cells were plated onto MRS agar containing erythromycin and incubated for 2 days at 37˚C. Recombinant plasmids were confirmed by PCR and double restriction enzyme digestion.

Plasmid stability test

The *in vitro* stability of pCycB21 and pTRKH2 was assessed in *L. plantarum* WCFS1. The transformants were cultured overnight in MRS broth supplemented with 15 μg/mL of erythromycin. The cultures were diluted with MRS broth to 10^6 cfu/mL. 100 μL of the diluted cultures were spread on MRS agar without antibiotic selection and incubated overnight at 30˚C. The next day, 100 colonies were randomly selected, patched onto MRS agar with and without erythromycin selection and incubated in the same condition. After the incubation, the numbers of colonies observed were calculated. The plasmid stability was expressed as percentage of colonies which retained the plasmid (i.e. the ratio between the number of erythromycin-resistant isolates and the total number of colonies grew on MRS agar without antibiotic).

Antimicrobial activity assay

The antimicrobial activity of the bacteriocin produced by LAB was evaluated using the well diffusion agar (WDA) method [43]. Briefly, cell free supernatants (CFS) of *L. plantarum* B21 and WCFS1 (pCycB21) were harvested from 15 mL overnight LAB culture by centrifugation at 4,000 × g for 20 min at 4˚C. The CFS was then concentrated 15-fold using an Amicon® Ultra-15 Centrifugal Filter Devices (Merck Millipore, Germany) and stored at 4˚C until used. To evaluate the antimicrobial activity of plantacyclin B21AG, MRS agar plates supplemented with 10 μg/mL of erythromycin were seeded with 10^6 cfu/mL of *L. plantarum* WCFS1 (pTRKH2), used as the indicator strain. Wells were made in the agar using a sterile 8-mm cork borer. One hundred microliters of the 2-fold serial diluted CFS was then loaded into the wells and the plates were incubated at 30˚C for 16–18 h. Antimicrobial activity was expressed as arbitrary unit (AU/mL) using the following equation, a^b x 100, where "a" is the dilution factor, "b" is the last dilution showing an inhibition zone of at least 2 mm in diameter [44].

Extraction of plantacyclin B21AG with 1-butanol

Plantacyclin B21AG was purified from *L. plantarum* B21 and *L. plantarum* WCFS1 (pCycB21) using 1-butanol as described by Abo-Amer [45] with the following modifications: the concentrated CFS was mixed with ½ volume of water-saturated butanol for 20 s. The mixture was incubated at room temperature for 10 min to allow phase separation before centrifugation at 10,000 × g for 10 min. The butanol phase was transferred to a clean 1.5 mL tube whilst the aqueous phase was subjected to an additional butanol extraction. The two butanol fractions containing plantacyclin B21AG were combined and the solvent was removed using a freeze dryer (FDU-8612, Operon Co. Ltd, Korea). The lyophilised protein was dissolved in 20 mM sodium phosphate buffer (pH 6.0).

Mass spectrometry analysis

The protein was subjected to matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analyses as described by Vater et al. [46]. The MALDI-TOF mass spectra were recorded using an Autoflex Speed MALDI-TOF instrument (Bruker, Germany) containing a 355 nm Smartbeam II laser for desorption and ionization. 10 mg of α-cyano-4-hydroxycinnamic acid dissolved in 70% acetonitrile (ACN) containing 0.1% (v/v) trifluoroacetic acid (TFA) was used as matrix solution. Five microliters of bacteriocin samples were mixed with equal volume of matrix solution and 1 μL of the mixture was spotted onto the target, air dried and measured.

Plasmid copy number (PCN) determination by real-time PCR

The copy number of the native (pB21AG01) and recombinant (pCycB21) plasmids were determined using real-time PCR according to Škulj et al. [47]. A 5-fold serial dilution of total DNA extracted from *L. plantarum* B21 was used for the standard curves (final 1 ng/μL to 0.0016 ng/μL). Real-time PCR reactions were performed in 12 μL mixtures containing 1 x SensiFAST SYBR No-ROX mix (Bioline, Australia), 400 nM of each forward and reverse primer (Table 2) and 1 μL of DNA. The alanine racemase gene (*alr*), a single copy, chromosomal gene from *L. plantarum* WCFS1, was selected as the reference gene (GeneBank Accession No. AL935253) whilst the bacteriocin structural gene (*orf19*) was chosen as the target for detection of the recombinant plasmid pCycB21. The replication (*rep*) gene was used as the target to detect pTRKH2 in WCFS1. Separate reactions were prepared for the detection of chromosomal and plasmid amplicons. All reactions were performed in duplicate using the Rotor-Gene^{TM} Q (Qiagen, Germany). Thermocycling conditions were: initial denaturation for 3 min at 95˚C, followed by 40 cycles of 5 s at 95˚C, 10 s at 55˚C and 20 s at 72˚C. Fluorescence signal was acquired at the end of each 72˚C step.

The slope of the relative standard curve with a condition that $r^2 > 0.99$ was used to calculate the amplification efficiency (E) using Eq (1).

$$\begin{aligned} E &= 10^{(-1/\text{slope})} \\ E(\%) &= (10^{(-1/\text{slope})} - 1) \text{ x } 100 \end{aligned} \quad (1)$$

The PCN was calculated based on Eq 2 using efficiency (E) and Ct values for both chromosomal (c) and plasmid (p) amplicons.

$$PCN = (Ec)^{Ctc}/(Ep)^{Ctp} \quad (2)$$

Results

Sequence analysis of pB21AG01

Sequence analysis revealed that *L. plantarum* B21 (GenBank Accession No. CP010528) harboured at least two cryptic plasmids, designated as pB21AG01 (GenBank Accession No.

Table 2. Primers used for plasmid copy number detection with real-time PCR.

Target gene	Location	Name	Sequence 5' → 3'	Amplicon size
alr	Chromosome	*alr*F	TGGGACGAATCGGGTTTCAG	208 bp
		*alr*R	GACACGTGGACATAGCGTGG	
orf19	pB21AG01	*orf19*F	CTGCAGCCTGCAGGGTTCAACCTCCTTTCTGAC	277 bp
		*orf19*R	GGTGGTCCTGCAGGCCTAACCTGCTACGATATGC	
rep	pTRKH2	*rep*F	CGCTCAATCACTACCAAG	102 bp
		*rep*R	CTCGGAAGTCAGAACAAC	

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CP025732) and pB21AG02 (GenBank Accession No. CP025733). For the purpose of this study, we focused our analysis on pB21AG01 as it was found to encode the genes responsible for the production of a circular bacteriocin. Plasmid profile analysis revealed that pB21AG01 is a 20,429 bp circular DNA molecule with a GC content of 37.3%. Twenty-six open reading frames (ORF) were identified (Table 3). The predicted proteins encoded by 14 ORFs were

Table 3. Putative genes and their proposed function deduced from the amino acid sequences of pB21AG.

Gene	Codon		No. of amino acids	Best homolog, GenBank Accession No. [organism]	% Identity (No. of amino acids overlapping)	Proposed function of gene product
	Start	Stop[a]				
orf1	2150	156C	664	Hypothetical protein, WP_057741928.1 [*Lactobacillus capillatus*]	97 (643)	Hypothetical protein
orf2	2808	2143C	221	DNA replication and relaxation protein, WP_057741930.1 [*Lactobacillus capillatus*]	93 (206)	Plasmid replication and relaxation
orf3	3590	3477C	37	No significant similarity		Hypothetical protein
orf4	4239	4030C	69	Hypothetical protein, WP_053266991.1 [*Lactobacillus plantarum*]	92 (55)	Hypothetical protein
orf5	5585	4599C	328	Hypothetical protein LVISKB P8-0006, BAN08211.1 [*Lactobacillus brevis* KB290]	92 (301)	Hypothetical protein
orf6	5748	5885	45	No significant similarity		Hypothetical protein
orf7	6592	5909C	227	Helix-Turn-Helix DNA binding domain of transcription regulators from the MerR superfamily, WP_057741904.1 [*Lactobacillus capillatus*]	85 (193)	Transcriptional regulator
orf8	7581	6667C	304	Hypothetical protein, WP_057741908.1 [*Lactobacillus capillatus*]	79 (246)	Hypothetical protein
orf9	8205	7597C	202	Hypothetical protein, WP_057741914.1 [*Lactobacillus capillatus*]	94 (189)	Hypothetical protein
orf10	9312	8230C	360	Cell wall hydrolase, WP_057741916.1 [*Lactobacillus capillatus*]	98 (352)	Hydrolysis of beta-1,4-linked polysaccharides
orf11	11112	9313C	599	Domain of unknown function DUF20, WP_057741917.1 [*Lactobacillus capillatus*]	90 (558)	Hypothetical protein
orf12	11484	11125C	119	Hypothetical protein, WP_053266985.1 [*Lactobacillus plantarum*]	93 (111)	Hypothetical protein
orf13	11657	11481C	58	Hypothetical protein FC81_GL002105, KRL03443.1 [*Lactobacillus capillatus*]	90 (52)	Hypothetical protein
orf14	13984	11657C	775	AAA-like domain containing a P-loop motif, KRL03444.1 [*Lactobacillus capillatus* DSM_19910]	99 (768)	Conjugative transfer
orf15	14588	14031C	185	TcpE family, WP_003688369.1 [*Lactobacillus mali*]	93 (172)	Conjugative transfer
orf16	14933	14601C	110	Hypothetical protein, WP_053266999.1 [*Lactobacillus*]	95 (104)	Hypothetical protein
orf17	15908	14949C	319	Conjugative transposon protein TcpC, WP_003688364.1 [*Lactobacillus mali*]	91 (290)	Conjugative transfer
orf18	16272	15979C	97	Hypothetical protein, WP_053266998.1 [*Lactobacillus*]	95 (92)	Hypothetical protein
orf19	16521	16796	91	Plantaricyclin A precursor, PlcA, PCL98053.1 [*Lactobacillus plantarum*]	88 (67)	Bacteriocin production
orf20	16888	17361	157	Plantaricyclin A immunity protein, PlcD, PCL98052.1 [*Lactobacillus plantarum*]	94 (147)	Involved in immunity to Plantaricyclin A
orf21	17364	17528	54	Plantaricyclin A immunity protein, PlcI, PCL98051.1 [*Lactobacillus plantarum*]	89 (48)	Involved in immunity to Plantaricyclin A
orf22	17548	18231	227	ABC transporter ATP-binding protein, PlcT, PCL98050.1 [*Lactobacillus plantarum*]	95 (215)	Transport
orf23	18234	18878	214	ABC-2 transporter permease, PlcE, PCL98049.1 [*Lactobacillus plantarum*]	94 (202)	Transport
orf24	18881	19402	173	Plantaricyclin A related protein, PlcB, PCL98048.1 [*Lactobacillus plantarum*]	90 (155)	Involved in Plantaricyclin A production
orf25	19567	19397C	56	Plantaricyclin A related protein, PlcC, PCL98047.1 [*Lactobacillus plantarum*]	95 (53)	Involved in Plantaricyclin A production
orf26	20229	19696C	177	Transposase DDE domain, CCB82565.1 [*Lactobacillus pentosus* MP-10]	100 (177)	DNA transposition

[a] C: complementary sequence

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homologous to proteins with known or predicted functions whilst the remaining 12 ORFs were either homologous to hypothetical proteins lacking functional predictions or had no significant homology with any protein sequences in the GenBank databases.

Seven ORFs were predicted to encode genes putatively responsible for the production, immunity, and transport of plantacyclin B21AG (*orf19* –*orf25*) [30]. *Orf19* showed 86% identity to plantaricyclin A precursor (PlcA), a circular bacteriocin produced by *L. plantarum* (PCL98053.1), presumably the structural gene responsible for the production of plantacyclin B21AG. It encodes 91 amino acids consisting of a 33 amino acid leader peptide and a 58 amino acid bacteriocin mature peptide, which is 86% identical to *plcA*, the structural gene of plantaricyclin A [25]. *Orf20* and *orf21* are 94% and 89% identical to *plcD* (PCL98052.1) and *plcI* (PCL98051.1), putatively involved in immunity to plantaricyclin A. *Orf22* and *orf23* are possible transporters of the plantacyclin B21AG. *Orf22* showed 95% identity to *plcT*, a gene encoded for ABC transporter ATP-binding protein in *L. plantarum* (PCL98050.1) whilst *orf23* is 94% identical to *plcE*, an ABC-2 transporter permease encoding gene in *L. plantarum* (PCL98049.1), respectively. *Orf24* and *orf25* are predicted as plantaricyclin A-related proteins, putatively involved in the production of the circular bacteriocin. Amino acid sequence comparison with gassericin A revealed that *orf20*, *orf24* and *orf25* may be membrane associated proteins, but their roles in bacteriocin production/immunity remain unknown [48]. Transmembrane analysis using TMHMM revealed the presence of 2 transmembrane domains in *orf19*, *orf21* and *orf25*. *Orf20* and *orf23* and *orf24* contain 4, 6 and 5 transmembrane domains, respectively. This result is consistent with the properties of the proteins involved in gassericin A production. No transmembrane domain was predicted for *orf22*.

Although no *rep* gene or direct repeats were found in pB21AG01, *orf2* showed 93% identity to a protein in *L. capillatus* (WP_057741930.1) which is essential for plasmid replication and relaxation. In addition, *orf7* is predicted to be a Helix-Turn-Helix DNA binding domain of transcription regulators from the MerR superfamily, accession number WP_057741904.1 [*Lactobacillus capillatus*] [39]. They have been shown to mediate responses to environmental stress including exposure to heavy metals, oxygen radicals and antibiotics or drugs in a wide range of bacterial genera [49]. *Orf10* is predicted as cell wall hydrolase, accession number WP_057741916.1 [*Lactobacillus capillatus*]. It is putatively involved in the hydrolysis of beta-1,4-linked polysaccharides [39]. *Orf26* is 100% identical to the pfam01609 transposase DDE domain of *Lactobacillus pentosus* MP-10 (CCB82565.1) which is essential for efficient DNA transposition. Although we did not find any *tra* genes responsible for bacterial conjugation in pB21AG01, three ORFs were annotated as genes related to plasmid mobilisation. *Orf15* and *orf17* showed 93% and 91% identity to genes encoded for the conjugative transposon proteins TcpE (WP_003688369.1) and TcpC (WP_003688364.1) of *L. mali*, respectively. *Orf14* is 99% identical to the pfam 12846 AAA-like domain containing a P-loop motif from *L. capillatus* DSM_19910 (KRL03444.1), putatively involved in conjugative transfer [39].

Cloning of the B21AG gene cluster and LAB transformation

The 3,424 bp sequence corresponding to seven genes putatively involved in plantacyclin B21AG production, secretion and immunity was cloned into the pTRKH2 shuttle vector. The resulting plasmid, pCycB21, transformed into *L. plantarum* WCFS1 is detailed in Fig 1.

The pTRKH2 and pCycB21 plasmids were electrotransformed into *L. plantarum* WCFS1 with efficiencies of 2.4 x 10^2 and 3.4 x 10^2 transformants per μg DNA, respectively. Addition of glycine in the growth medium inhibits formation of cross-linkages in the cell wall where $_L$-alanine is replaced by glycine, thereby weakening the cell wall and facilitating DNA update by the cells [50, 51].

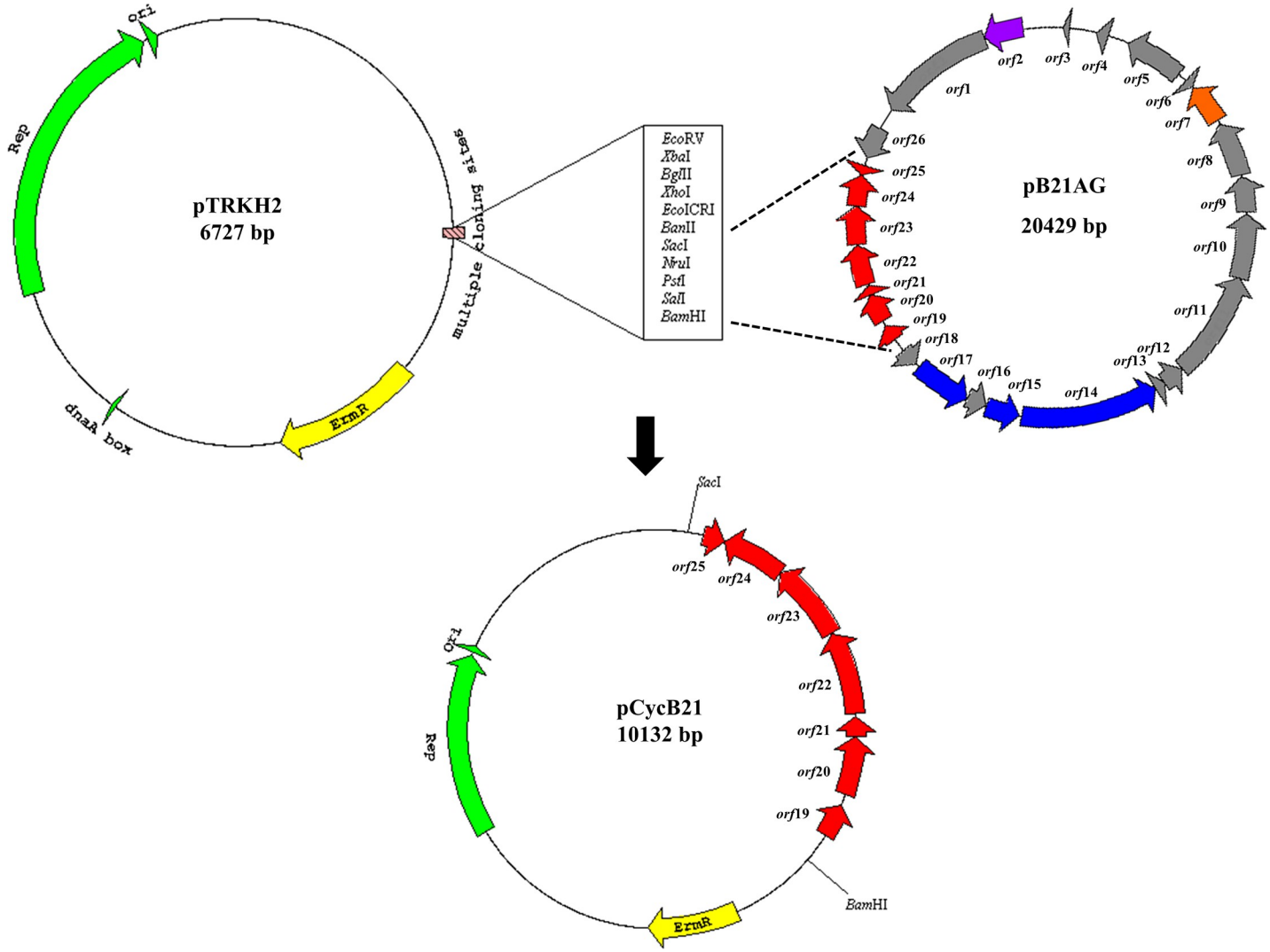

Fig 1. Map of pTRKH2, pB21AG01 and pCycB21. Top left: The plasmid pTRKH2 has an origin of replication from plasmid P15A and a replication gene (green arrows) for replication in both *E. coli* and Gram-positive bacteria; an erythromycin resistance gene (ErmR, yellow arrow) for selection in *E. coli* and LAB; and a multiple cloning site (MCS). Top right: Map of pB21AG01 containing 26 open reading frames, with seven ORFs corresponding to bacteriocin production, immunity and transportation (red arrows); a plasmid replication and relaxation gene (purple arrow); a transcription regulator (orange arrow), three conjugation transfer genes (blue arrows) and 14 other ORFs (grey arrows). Bottom: Map of pCycB21 harbouring seven bacteriocin-associated genes cloned into *Sac*I and *Bam*HI restriction sites.

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Plasmid stability assay

The plasmid stability assay was performed on plasmid pCycB21 and pTRKH2 on MRS agar with and without antibiotic selection. pTRKH2 exhibited higher plasmid stability, with 68% of the cells retained the plasmid after 20 generations. In contrast, pCycB21 is less stable in the host *L. plantarum* WCFS1, with only 34% of the transformants maintained the plasmid. These results show that the cloned bacteriocin gene cluster has increased plasmid instability, and erythromycin is required to maintain selection of pCycB21 in the recombinant host.

Assay of plantacyclin B21AG expression

Antimicrobial activity of the wild type *L. plantarum* B21 and recombinant host *L. plantarum* WCFS1 (pCycB21) were assayed using the well diffusion agar (WDA) method with *L.*

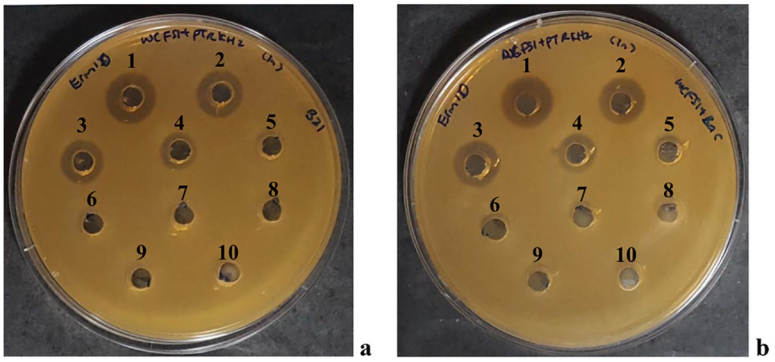

Fig 2. Antimicrobial activity of a two-fold serial dilution of plantacyclin B21AG secreted by (a) *Lactobacillus plantarum* B21 and (b) WCFS1 harbouring pCycB21. *L. plantarum* WCFS1 (pTRKH2) was used as the indicator strain. Numbers above the wells correspond to the CFS dilution in each well. 1, Undiluted CFS; 2, 1:2 dilution of CFS; 3, 1:4 dilution of CFS; 4, 1:8 dilution of CFS; 5, 1:16 dilution of CFS; 6, 1:32 dilution of CFS; 7,1:64 dilution of CFS; 8, 1:128 dilution of CFS. Well 9 (indicator strain) and well 10 (MRS broth) are negative controls.

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plantarum WCFS1 (pTRKH2) as indicator strain. To eliminate the effect of acid production, the pH of cell free supernatants was neutralised to pH 6.5. Both wild type and recombinant hosts were found to produce inhibition zones against the indicator strain up to 1:8 dilution (Fig 2). The antimicrobial activity was calculated as 800 AU/mL for both strains. This indicates that the inhibitory activity is not due to acid production but to an antimicrobial substance secreted into the broth [52]. The CFS of control cultures containing WCFS1 (pTRKH2) did not show any inhibitory activity, confirming that the recombinant plasmid pCycB21 was responsible for the antimicrobial activity.

Mass spectrometry analysis

The plantacyclin B21AG produced by the wild type B21 and recombinant host WCFS1 (pCycB21) was purified by extraction into butanol. MALDI-TOF-MS analysis revealed a major peptide of molecular mass of 5663.9 Da, essentially identical to the plantacyclin B21AG produced by the wild type *L. plantarum* B21 (5664.7 Da) (Fig 3). No major peaks were observed for WCFS1 transformed with the shuttle vector pTRKH2, corroborating the results from the functional expression assay described previously (Fig 2, well 9).

Copy number of pB21AG01 and pCycB21

The relative copy number of pB21AG01 and pCycB21 was determined by real-time PCR using the single copy alanine racemase (*alr*) as reference gene. In our experiment, the standard curves obtained for *alr*, *orf19* and *rep* were linear ($R^2 > 0.99$) over the range tested; whilst the amplification efficiency for all experiments ranged between 90–102%, which is within the acceptable range (90–110%) [53]. Analysis of the results revealed that approximately 7.60 ± 0.79 copies per cell of pB21AG01 was detected in *L. plantarum* B21 whilst pCycB21 in *L. plantarum* WCFS1 was present at a noticeably lower level of just 0.51 ± 0.05 copies, indicating that only half the cells carry the recombinant plasmid. In contrast, the copy number of the shuttle vector only, pTRKH2 in *L. plantarum* WCFS1 was high at 25.19 ± 2.68 copies per cell (average for two clones ± standard deviation) per chromosome equivalent.

Discussion

Several lactic acid bacteria species have been recognised as probiotics that possess important traits such as the production of bacteriocins and organic acids, adhesion to host cells, and

resistance to antibiotics and heavy metals [54, 55]. A number of native plasmids that encode these probiotic traits have been sequenced from *L. plantarum* [54, 56], *L. salivarius* [57] and *L. fermentum* [55]. In this study, seven bacteriocin-associated genes were found to be located on a 20 kb native plasmid, pB21AG01 in *L. plantarum* B21. The structural gene of plantacyclin B21AG (*orf19*) was found to be similar (86%) to plantaricyclin A [25]. They are both 58 amino acid in length. Comparison of the amino acid sequences between the two revealed that they both comprise of 72% hydrophobic residues, and plantaricyclin A contains more uncharged/polar residues (21%) than plantacyclin B21AG (19%). The high hydrophobicity may imply the importance of hydrophobic interaction in the mechanism of circularisation [58] or the mediation of initial contact between the peptides and bacterial membrane [13, 26]. Notably, plantacyclin B21AG has an overall net charge of +3 as it contains four basic residues (one arginine, one histidine and two lysine) and one acidic residue (aspartic acid). In contrast, plantaricyclin A contains one histidine and two lysine, and one aspartic acid, imparting a lower net charge of +2. These positively charged residues at the surface of the peptides are thought to be involved in the initial electrostatic interaction between the circular bacteriocins and the negatively charged phospholipid bilayer of the target bacterial membrane [3, 26]. The variation of the overall positive charge may imply a difference in the mode of action, as inferred by the different antimicrobial spectrum between the two bacteriocins [25, 30].

No replication protein (repB) or initiator replication family protein (repA) was found in pB21AG01. However, a DNA replication and relaxation conserved domain was detected in *orf2*. We could not detect any clear repeats in the region upstream of *orf2*, suggesting that the plasmid may replicate through a mechanism yet to be determined [59]. In addition, we identified two *tcp* loci, TcpE (*orf15*) and TcpC (*orf17*), which are involved in the transfer of conjugative plasmid, pCW3 from *Clostridium perfringens*. TcpE was shown to play a role in the formation of Tcp transfer apparatus in the Gram-positive *C. perfringens* [60]. TcpC was identified as a bitopic membrane protein, where membrane localisation is important for its function, oligomerisation, and interaction with other conjugation proteins [61]. Bantwal et al. [62] proposed that TcpC may initiate accumulation of peptidoglycan hydrolase at the cell wall of *Clostridium*, resulting in degradation of peptidoglycan, and thus facilitate the formation of the transfer apparatus. Interestingly, a cell wall hydrolase (*orf10*) was also found in pB21AG01, suggesting that TcpC may play a role in promoting the hydrolysis of *Lactobacillus* cell wall and subsequently the transfer of pB21AG01. However, we did not transfer the plasmid by mating because we could not determine a full set of genes responsible for the mobilisation of the plasmid. pCW3 has a novel conjugation region consisting of 11 genes encoding the Tcp proteins (TcpA, TcpB, TcpC, TcpD, TcpE, TcpF, TcpG, TcpH, TcpI, TcpJ and TcpM) [60, 63]. Several studies have demonstrated that TcpA, TcpD, TcpE, TcpF and TcpH are essential to form the conjugation complex. Moreover, we could not detect any antibiotic resistance and/or heavy metal resistance genes in pB21AG01, which could be used as natural selection markers if we were to transfer the plasmid by mating. Due to the lack of *tcp*-encoded proteins and selection markers, we decided to construct a recombinant plasmid, including the plantacyclin B21AG operon and a selection marker, and transfer the plasmid by electroporation.

Electroporation is an effective method to transfer plasmid DNA into LAB to enhance their probiotic functionality, or to secrete therapeutic proteins into the culture medium for human and animal health [64]. However, the success rate of LAB transformation is extremely low compared to *E. coli* due to various restriction modification (RM) systems encoded by the host. RM systems are required to protect bacteria from foreign DNA such as genetically transferred plasmid DNA or bacteriophage [64, 65]. Since DNA manipulation is easier in *E. coli* than in *Lactobacillus*, we have built the recombinant plasmid in the shuttle vector pTRKH2, followed by propagating the plasmid in *E. coli* to obtain sufficient amount of plasmid DNA for LAB

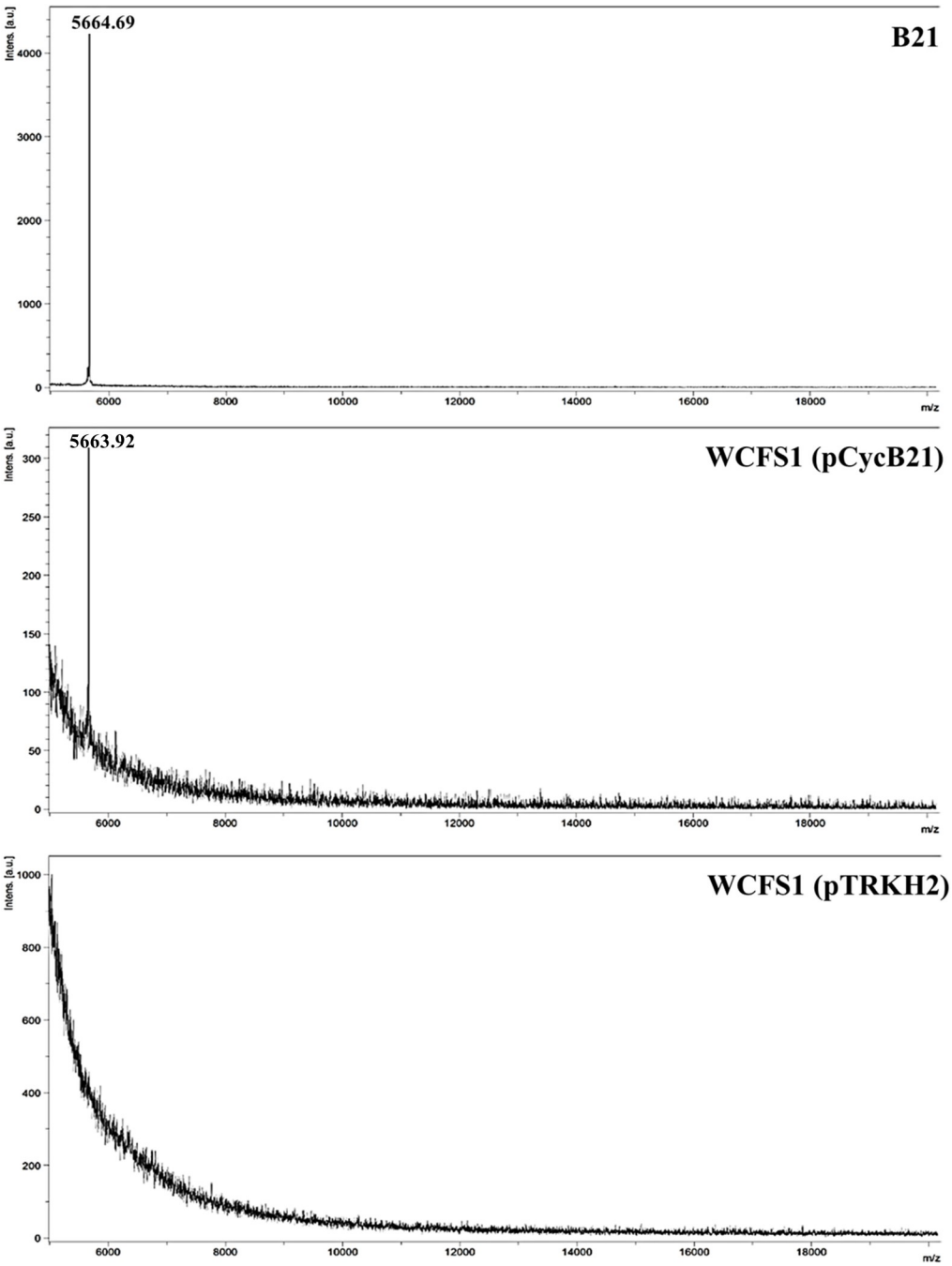

Fig 3. MALDI-TOF-MS spectrum of plantacyclin B21AG. A single peak was detected at molecular mass of 5664.69 for B21 and 5663.92 for WCFS1 (pCycB21). No major peaks were observed for WCFS1 (pTRKH2).

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transformation. Numerous attempts at electrotransformation were performed according to various protocols described in the literature but without any success. The parameters for electrotransformation that we have tried included varying the percentage of glycine added to LAB culture prior to pelleting the cells; addition of PEG_{1500} and EDTA in the washing steps; various concentrations of plasmid DNA; different electroporation buffers and diverse combinations of voltage (V), capacitance (μF) and resistance (ohm). The final modified version of the method

described [42], that we eventually had success with is described in the materials and methods section.

A few attempts have been made to heterologously expressed bacteriocins in different LAB species because they promise a food grade background, where the expression of bacteriocins would enhance their probiotic functionality [28, 66]. However, all of these peptides that have been successfully expressed heterologously belong to the class II bacteriocins. To date, only one circular bacteriocin, plantaricyclin A from *L. plantarum* NI326, has been successfully cloned into a nisin-inducible plasmid and expressed in *L. lactis* pNZPlc. Both the recombinant host and the wild type producer exhibited similar level of antimicrobial activity [25], indicating that circular bacteriocin can be heterologously expressed in other LAB species. Our results are in accordance with Borrero et al. [25], where both native producer and the recombinant host expressed similar antimicrobial activity, up to 800 AU/mL. This result is also confirmed by a single peak observed in our mass spectrometry analyses. In contrast, *L. plantarum* WCFS1 transformed with the empty vector pTRKH2 produced no bacteriocin activity. These results indicate that the bacteriocin activities observed are due to the cloned genes (*orf19 –orf25*). We demonstrated that a 3.4 kb plasmid region of *L. plantarum* B21 is sufficient for functional expression of plantacyclin B21AG. However, our attempt to transform pCycB21 into *Lactobacillus agilis* La3, a type of LAB found to efficiently colonise chicken gastrointestinal tract (GIT) [67], did not result in heterologous expression of the bacteriocin. This result suggests that the expression of recombinant protein in LAB is species-specific. One possible explanation is that the native promoter used to express the plantacyclin B21AG is specific to *L. plantarum*, and a promoter from *L. agilis* may be required for heterologous expression. Although bacterial promoters share similar features, promoter strength is strain- and context-specific, and can vary significantly within LAB [66, 68, 69].

The isolation and purification of bacteriocins from their LAB producers is often very time-consuming and labour intensive [70]. Many studies have been performed to heterologously express and overexpress the class II bacteriocins in *E. coli* to facilitate the production of these antimicrobial peptides. For instance, sakacin P, pediocin PA-1, divercin V41 and plantaricin NC8 have been successfully expressed in *E. coli* [70–73]. However, no circular bacteriocins have been successfully expressed in *E. coli*. Kawai et al. [74] tried to express a circular bacteriocin, gassericin A, in *E. coli* JM109 as a biotinylated fusion protein. However, a positive clone which accumulated the bacteriocin did not show any antimicrobial activity. Further treatment with factor Xa protease released the N-terminal leader peptide, resulting in an active unclosed gassericin A. The results indicate that expression of circular bacteriocins is host-specific, where a yet-to-be identified host-encoded peptidase is required to cleave the leader peptide, allowing the ligation of N- and C- terminal to happen [13, 75].

PCN analysis showed that the native pB21AG01 is a highly stable, low copy number plasmid in *L. plantarum* B21. pTRKH2 was selected as a shuttle cloning vector because it has been shown to be structurally stable in *L. vaginalis* Lv5, a common feature of the theta-replicating mechanism [67]. It also has good structural stability in *E. coli*, possibly due to the lack of a resolvase-encoding gene [35]. A plasmid stability assay and PCN analysis revealed that pTRKH2 is more stable than pCycB21 in *L. plantarum* WCFS1, indicating that the bacteriocin gene cluster may cause instability of the vector pTRKH2. Erythromycin selection is required to maintain the pCycB21 in *L. plantarum* WCFS1. One possible reason which may contribute to the instability of pCycB21 is plasmid incompatibility, where two plasmids containing the same origin of replication cannot co-exist stably in the cell. Plasmids that have growth advantages, such as faster replication and less toxicity will rapidly outgrow the other plasmids [76]. The host used in this study, *L. plantarum* WCFS1 is known to harbour three native plasmids size 1.9 kb, 2.4 kb and 36 kb [54]. Thus, the introduced bacteriocin gene cluster could be a plausible

reason for pCycB21 instability in *L. plantarum* WCFS1. Similarly, the copy number of pCycB21 is extremely low compared to the native plasmid pB21AG01. This suggests that other fitness factors present on the native plasmid pB21AG01 may also play a role in positive plasmid selection. For instance, apart from the immunity genes which are known to protect bacteriocin-producing strains against its own toxins, the gene encoding for ABC transporter also plays an important role. It has been shown to translocate the bacteriocin across the cytoplasmic membrane, thereby avoiding toxin accumulation in the host cells [77, 78]. In our case, the presence of a general ABC transporter could potentially stabilise pB21AG01 in *L. plantarum* B21. Despite the PCN variation between pB21AG01 and pCycB21, plantacyclin B21AG was expressed at a similar level. The production of plantacyclin B21AG would depend on plasmid stability and copy number differences between pB21AG01 and pCycB21, but more likely, might be caused by the promoters used to drive gene expression [7]. We have cloned the native promoter from pB21AG01 into pCycB21, presumably resulting in similar levels of plantacyclin B21AG production. In the future, inducible or controlled promoters may be tested to optimise heterologous production of plantacyclin B21AG [66].

In summary, circular bacteriocins are thought to have more potential to form the next generation of biopreservatives as a consequence of their stability and activity [8]. The ability to transfer vectors harbouring the plantacyclin B21AG gene cluster into an industry standard probiotic *L. plantarum* WCFS1 highlights its biotechnological interest for the overproduction of the antimicrobial peptide with high antimicrobial activity against food-borne pathogens and unlocks the possibility of undertaking systematic structure function studies by the directed mutation and evolution of the structural gene.

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Methodology: Mian Chee Gor, Thi Thu Hao Van.

Project administration: Mian Chee Gor, Thi Thu Hao Van.

Resources: Aida Golneshin, Robert J. Moore, Andrew T. Smith.

Supervision: Thi Thu Hao Van, Robert J. Moore, Andrew T. Smith.

Validation: Mian Chee Gor.

Visualization: Mian Chee Gor.

Writing – original draft: Mian Chee Gor.

Writing – review & editing: Mian Chee Gor, Thi Thu Hao Van, Robert J. Moore, Andrew T. Smith.

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