TITLE: Impact of Environmental Factors on Bacteriocin Promoter Activity in Gut-Derived Lactobacillus salivarius

AUTHORS: Guinane CM, Piper C, Draper LA, O'Connor PM, Hill C, Ross RP, Cotter PD.

Impact of environmental factors on bacteriocin promoter activity in gut-derived *Lactobacillus salivarius*

Caitriona M. Guinane[1], Clare Piper[1], Lorraine A. Draper[2,3], Paula M. O'Connor[1,2], Colin Hill[2,3], R. Paul Ross[2,4], Paul D. Cotter[1,2]

Teagasc Food Research centre, Moorepark, Fermoy, Co Cork[1]; APC Microbiome Institute , University College Cork[2]; School of Microbiology, University College Cork[3], School of Science, Engineering & Food Science, University College Cork, Cork, Ireland[4].

To whom correspondence should be addressed: paul.cotter@teagasc.ie

ABSTRACT

Bacteriocin production is regarded as a desirable probiotic trait that helps colonisation and persistence in the gastrointestinal tract (GIT). Strains of *Lactobacillus salivarius,* a species associated with the GIT, are regarded as promising probiotic candidates and have a number of associated bacteriocins documented to date. Among these include multiple class IIb bacteriocins (salivaricin T, salivaricin P and ABP-118) and the class IId bacteriocin bactofencin A, that show activity against medically important pathogens. However, the production of a bacteriocin in laboratory media does not assure production under stressful environmental conditions, such as those encountered within the GIT. To allow this issue to be addressed, the promoter regions located upstream of the structural genes encoding the aforementioned *L. salivarius* bacteriocins were fused to a number of reporter proteins (green fluorescent protein [GFP], red fluorescent protein [RFP] and luciferase [Lux]). Of these only transcriptional fusions to GFP generated signals of sufficient strength to study promoter activity in *L. salivarius*. While analysis of the class IIb promoter regions revealed relatively weak GFP expression, assessment of the promoter of the anti-staphylococcal bacteriocin bactofencin A indicated a strong promoter that is most active in the absence of the antimicrobial peptide and positively induced in the presence of mild environmental stresses, including simulated gastric fluid. Taken together this data provides information on factors that influence bacteriocin production, which will assist in developing strategies to optimise the *in vivo* and *in vitro* production of these antimicrobials.

INTRODUCTION

Bacteriocins are a heterogeneous family of small, ribosomally synthesised peptides with antimicrobial activity produced by many bacterial species (1-3). These antimicrobials can have a broad or narrow spectrum of activity and have considerable potential as agents in food preservation and in biomedical applications. Bacteriocin production is considered an important trait of gut-derived bacteria influencing microbial populations within the intestinal tract (review see (4)). *Lactobacillus salivarius* is a species associated with the GIT with many associated probiotic traits. *L. salivarius* strains are frequently producers of class IIa, IIb and IId unmodified bacteriocins (5-9). Class IIa bacteriocins are generally designated as pediocin-like peptides, class IIb comprise the two-component unmodified peptides and class IId bacteriocins are often categorised on the basis of their dissimilarity to other class II peptides (10).

L. salivarius NCIMB 40829 (**LSUCC118™**) is an extensively studied strain and produces the class IIb two-peptide bacteriocin ABP-118 (11). The *in vivo* functionality of the ABP-118 bacteriocin and its effectiveness in eliminating a GIT pathogen in a mouse model has been previously demonstrated (12). Closely-related variants of ABP-118, such as salivaricin P, and indeed other two-peptide bacteriocins, such as salivaricin T, have also been isolated from several intestinal *L. salivarius* strains (7, 13). Production of and immunity to these bacteriocins relies on the coordinated expression of at least 12 genes (8, 11, 13). More recent work has led to the identification of a novel class IId bacteriocin, bactofencin A, produced by porcine intestinal isolates of *L. salivarius* (9). This bacteriocin gene cluster consists of just 4 genes including the structural peptide, BfnA, which shows little identity to previously isolated bacteriocins but shares some similarity with eukaryotic cationic peptides. This bacteriocin was demonstrated to have antimicrobial activity against medically important pathogens, including *Staphylococcus aureus* (9). The level of production of bactofencin A

and indeed many other bacteriocins *in vivo* and to how their production is influenced by stressful environmental conditions are as yet unknown. This is despite the fact that it has been documented that bacteriocin production can be sensitive to environmental changes and parameters including temperature, pH and growth medium (14-16). Research of this nature is critical with respect to the successful use of bacteriocin producing strains for food and/or medical applications.

With respect to what is known already, it is clear that the regulation of bacteriocin production can be complex and in some cases involves a quorum sensing cell-density dependant mechanism that relies on a pheromone-like peptide and a cognate two component regulatory system (17). The inducing peptides (IP), thought to be produced at a low basal level in early growth, can reach a critical threshold concentration either due to an accumulative process or by increased production elicited by an environmental stimulus. Once the required IP level is reached, the signal is processed by the two component system, which binds to the promoters of the bacterial structural genes to allow bacteriocin production (17). These regulatory mechanisms and the promoter elements involved have been studied in depth in the cases of plantaricin (18, 19) and sakacin (20, 21) bacteriocin gene clusters. In some cases, most notably in lantibiotic (class I) gene clusters, the bacteriocin itself can function as the inducing peptide (22).

Of the salivaricins that are the focus of our studies, the 3 class IIb non-lantibiotic bacteriocins (ABP118, Salivaricin P and T) are predicted to have a IP-associated regulatory mechanism similar to that described above (11), whereas the gene cluster associated with the class IId bacteriocin bactofencin A does not contain an obvious regulatory mechanism (9). With a view to identifying the environmental factors that influence the production of these bacteriocins, the putative bacteriocin promoter regions were fused to a reporter gene to detect promoter activity under various environmental conditions. More specifically, a stable

expression system using the Green Fluorescent Protein (GFP) was established and the promoter.*gfp* fusions were monitored in *L. salivarius* bacteriocin-producing backgrounds. Promoter activity was assessed under a number of environmental conditions, some of which simulate the stressful environment of the GIT. This knowledge will allow the development of strategies to optimise the production of these bacteriocins *in vivo* and *in vitro* and provide valuable fundamental insights that will facilitate similar experiments with other bacteriocin-producing microbes in the future.

MATERIALS AND METHODS

Bacterial strains and culture conditions

The *Escherichia coli* and *L. salivarius* strains and plasmids used in this study are listed in Table 1. *E. coli* strains were grown aerobically in Luria-Bertani (LB) medium at 37°C. *L. salivarius* strains were cultured under anaerobic conditions in MRS (Difco Laboratories, Detroit, MI) at 37°C for 24-48 h, except for fluorescence expression analysis where cells were grown statically at 37°C, to achieve microaerobic conditions. Ampicillin (50 µg/mL) and Chloramphenicol (Sigma-Aldrich, St. Louis, MO, USA) was added at 10 µg/mL and 5 µg/mL for *E. coli* and *L. salivarius* strains respectively.

In silico analysis of putative promoter regions

The bacteriocin gene clusters of salivaricin P (13), salivaricin T (8), ABP-118 (11) and bactofencin A (9) were analysed using Artemis software (23). Regions upstream of the structural peptides and prepeptides were investigated for putative promoter regions using BPROM (24), Virtual Footprint (version 3.0) (25) and by manual annotation of the operons. Direct repeats were searched for using the Tandem Repeats Finder software (26).

DNA manipulations

Primers used for PCR were purchased from Sigma-Aldrich (St. Louis, MO, USA) and are listed in Table S1 in supplemental material. Platinum Taq DNA polymerase (Life technologies, Carlsbad, CA, USA) or MyTaq Red mix (Bioline, London, UK) was used for PCR amplifications. Restriction enzymes, Klenow DNA polymerase I and T4 DNA ligase were all purchased from Roche (Manheim, Germany) and used as outlined in manufacturer's instructions. PCR purification, gel extraction and plasmid preparation kits were obtained from Qiagen (Venlo, Netherlands) and used as specified by the manufacturers. The genomic DNA of *L. salivarius* strains was extracted as previously described (27).

Construction of expression plasmids

The pNZ44 plasmid expressing reporter proteins GFP, DsRed (Red fluorescent protein, RFP) and the Lux (luciferase) system were created as follows. The *gfp+* gene including a ribosome binding site (RBS) was amplified from the plasmid pEVS*gfp+* (28) using the primers *gfp+*For and *gfp+*Rev (Table S1) containing the *Pst*I and *Xba*I restriction sites. The cloning vector pNZ44 and *gfp* insert were digested with the restriction enzymes *Pst*I and *Xba*I, ligated together and the resulting pNZ44.*gfp+* plasmid was transformed into chemically competent *E. coli* DH10B cells (Life Technologies, Calrsbad, CA, USA) and subsequently into *L. salivarius* cells. The DsRed reporter gene (*rfp*) was amplified from the pDsRed-Express vector (Clontech, Mountain view, CA, USA) using the primers DsRed+For and DsRed+Rev (Table S1) to create the pNZ44.*rfp+* plasmid. These primers incorporated a RBS site and altered start codons to reduce the GC content at the beginning of the gene so as to increase the likelihood of translation initiation as previously recommended (29). The luciferase genes were amplified from the pP2lux plasmid (30) with the *lux+*For and *lux+*Rev primers and also cloned into the *Pst*I and *Xba*I sites of pNZ44 to create the pNZ44.*lux+* plasmid (Table 2). In

certain *gfp+* plasmids the p44 promoter was replaced with a constitutive lactobacilli promoter (p*cysK*). Subsequently, putative promoter regions for the bacteriocin operons bactofencin A, salivaricin P, T and ABP-118 were amplified from *L. salivarius* genomic DNA and using the *Bgl*II and *Pst*I restriction sites were cloned to create transcriptional fusions to the *gfp+* gene. Constructs created are outlined in Table 2; primers used to create the amplicons for cloning are described in Table S1. To generate the 151bp putative promoter region representing *sal*prom3, the sequence was synthesised using the gene synthesis service by GeneWiz/Sigma-Aldrich (St. Louis, MO, USA) including *Bgl*II and *Pst*1 restriction sites to facilitate cloning into the pNZ44.*gfp* plasmid (Fig. S1). Plasmids were transformed into chemically competent *E. coli* DH10B cells (Life Technologies, Carlsbad, CA, USA). Following verification of the integrity of the plasmid constructs, they were transformed into electrocompetent *L. salivarius* bacteriocin-producing backgrounds (*L. salivarius* DPC6502 (bactofencin A producer), *L. salivarius* DPC6488 (salivaricin T and salivaricin L (class IId bacteriocin producer (8)), *L. salivarius* DPC6189 (salivaricin P and bactofencin A producer) and *L. salivarius* NCIMB 40829 (**LSUCC118™**) (ABP-118 producer) to create a bank of *gfp+* strains (Table S2). *Lactobacillus salivarius* competent cells and electroporation procedures were performed as described previously (31). To create a promoter-less plasmid containing the *gfp* gene to serve as a negative control, the p44 promoter was removed from the pNZ44.*gfp* plasmid using the *Bgl*II and *Pst*I restriction sites. The plasmid ends were treated with Klenow DNA polymerase and subsequently ligated with T4 DNA ligase. The integrity of the constructs was confirmed by sequencing (Beckman Coulter Genomics, Takeley, UK).

Expression of reporter genes in *L. salivarius*

To detect fluorescence in *L. salivarius* strains containing the promoter.*gfp+* fusions, cells were grown overnight in broth at 37°C until stationary phase. Cells were harvested and

washed with phosphate-buffered saline (PBS) and subsequently the cell suspensions were analysed using an epifluorescence microscope (Olympus BX-51) equipped with a fluorescein isothiocyanate filter under the Olympus UPlanFl 100X oil Iris objective lens. Images were captured with a DP50 camera (Olympus Co., Toyko, Japan) and analysed with the Olympus analySIS software. To detect fluorescence from *L. salivarius* strains containing promoter.*rfp+* fusions, cells were prepared as above and analysed using the Olympus microscope equipped with a tetramethylrhodamine isothiocyanate filter for red emission.

To detect bioluminescence, overnight cultures were inoculated at 1-2.5% in fresh MRS broth for *L. salivarius* cells and in LB broth for *E. coli* cells and transferred in to 96-well plates, incubated and monitored on a Xenogen IVIS 100 system (Xenogen, Almeda, CA, USA) at 37°C. The levels of bioluminescence were determined in continuous imaging mode at high resolution with 5 min exposure times.

Growth and fluorescence assays in *gfp+* strains

Broth-based assays were performed by inoculating fully grown *L. salivarius* strains containing the promoter.*gfp+* gene fusions at 2% w/v in a volume of 0.2 mL of media (MRS) and grown statically at 37°C. The optical density at 600nm was monitored to determine bacterial growth or to detect GFP fluorescence (excitation 485nm, emission 520nm) in a Synergy 2 spectrophotometer (Bio-tek, Winooski, VT, USA) over a 20 h period. Data was recorded and plotted in relative fluorescence units (RFU).

Challenge assays to environmental stimuli and stresses

Stationary phase cells of *L. salivarius gfp+* strains were harvested by centrifugation and cell pellets were washed with PBS and resuspended in MRS broth. Cells were then inoculated at 2% w/v into 1mL MRS broth at pH6.5, pH6 and pH5.5 (adjusted with 1M HCL), into MRS

broth containing 0.25, 0.5% and 1% w/v NaCl concentrations or in to MRS broth containing 0.1, 0.2% and 0.3% porcine bile (Sigma-Aldrich, St. Louis, MO, USA). 0.2 mL aliquots were dispensed in to 96-well plates and monitored as described above.

To assess GFP expression from cells in response to the presence of target microbes, *gfp+* strains were inoculated at 1 x 10^9 CFU/mL in to MRS broth with a bacteriocin sensitive strain (*Lactobacillus delbrueckii* subsp. *bulgaricus* LMG 6901) at 1 x 10^7 CFU/mL. GFP expression was monitored in 96-well plates as described above. To assess promoter activity in the presence of bacteriocin inducing peptides (IP), the recognised IP for salivaricin P (MKFEVLTEKKLQVIVGGKQEGGTKTYDKVCRFKFLGICK) and the IP that is specific to both salivaricin T and ABP-118 (MKFEVLTEKKLQKIAGGATKKGGFKRWQCIFTFFGVCK) were synthesised using Microwave-assisted Solid Phase Peptide Synthesis (MW-SPPS) performed on a LibertyBlue^{TM} CEM microwave peptide synthesiser. Both peptides were synthesised on an Fmoc-L-Lys(BOC) HMBP-Chemmatrix resin (PCAS BioMatrixInc, Quebec, Canada) and purified using RP-HPLC on a Vydac C8 (10 u, 300A) column (Vydac, California, USA) and eluted using a 20-40% acetonitrile 0.1% TFA gradient over 40 minutes. The flow rate was 2.5 ml/min and eluent monitored at 214 nm. Fractions containing the desired molecular mass were identified using MALDI TOF Mass Spectrometry (Shimadzu Biotech, Manchester UK) and were pooled and lyophilised on a Genevac HT 4X (Genevac Ltd. Ipswich, UK) lyophiliser. Peptides were added to harvested cells in MRS broth at 10^{-4}-10^{-6}M and fluorescence was monitored over 20 h as described above.

Challenge assays to simulated gastric fluid

To assess GFP expression from cells exposed to simulated gastric fluid overnight cells were washed in PBS and subsequently resuspended in gastric fluid for 30 secs, 1 min and 5 min. Cells were then harvested and inoculated into MRS broth and fluorescence was detected as

described above. Simulated gastric fluid was made as previously described (32) consisting of NaCl (2.05 g/L), KH$_2$PO$_4$ (0.60 g/L), CaCl$_2$ (0.11 g/L) and KCl (0.37 g/L), adjusted to pH 2.0 using 1M HCl and autoclaved at 121°C for 15 min. Porcine bile (0.05 g/L), lysozyme (0.1 g/L) and pepsin (0.0133 g/L) were added as stock solutions prior to use. Components were obtained from Sigma-Aldrich (St. Louis, MO, USA).

RESULTS

Selection of a reporter system for *L. salivarius*

The GFP, RFP and Lux reporter systems were investigated to test their suitability as tools to study promoter activity in *L. salivarius*. Due to the potential usefulness of the Lux system with respect to facilitating *in vivo* detection (33), the creation of a luciferase reporter system in *L. salivarius* strains was targeted. The synthetic Lux operon, containing *luxABCDE*, encoding luciferase (LuxAB) and a fatty acid reductase complex (LuxCDE) was amplified from the pP2lux plasmid (34) and cloned after the constitutively expressed p44 promoter in the pNZ44 plasmid. Although notable bioluminescence was obtained in the *E. coli* host (Fig. 1), *L. salivarius* cells containing this vector did not generate bioluminescence of sufficient strength to merit its continued use.

DsRed is a popular reporter protein of RFP and often used as an alternative to GFP due to the generation of a more optimal emission spectrum for fluorescence within complex and live tissues (for review see (35)). The DsRed reporter protein is available commercially as part of a transcriptional fusion with *lacZ* (Clontech). However, when the *dsred* ORF is moved to another vector it is often not efficiently expressed and can develop more slowly than GFP (29, 36). To optimise the production of RFP, steps were taken in the oligonucleotide design to reduce the GC content at the start at the 5` end of the gene and a typical RBS ACGAGG was inserted 8 bp before the translational start site, as previously

suggested (29) (Table S1). Despite noticeable fluorescence in *E. coli,* upon transferring the pNZ44.*rfp* vector into *L. salivarius,* we were again unable to detect sufficient fluorescence from fully grown cultures to justify its continued use (Fig. 1).

GFP is a highly useful stable and species-independent fluorescent reporter and, unlike bioluminescent reporters, does not require the addition of specific substrates (other than molecular oxygen) for reaction efficiency. Following synthesis, GFP requires an autocatalytic reaction creating a fluorophore by oxidation (37). Previous work has established that GFP can be used as a reporter in *L. salivarius* cells (38). In this study the *gfp* gene with a corresponding RBS site (AGGAGG) was cloned downstream of the constitutive *Lactococcus lactis* p44 promoter and fluorescence was observed. The p44 promoter was replaced with a constitutive *Lactobacillus* promoter p*cysk* (from *L. salivarius* NCIMB 40829 (**LSUCC118^TM**)), and although the level of fluorescence observed was indeed higher, it was reasonably comparable with that detected with the p44 promoter and therefore continued use of the lactococcal promoter fusions as a positive control for GFP expression was sufficient (Table 2; Fig. 1).

Notably, although a very useful reporter *in vitro, in vivo* analysis using *gfp+ L. salivarius* cells were previously not successful due to background tissue, and indeed food, autofluorescence (unpublished data) and therefore *in vitro* tests were relied upon to investigate the impact of environmental stresses on promoter activity.

In silico **analysis of bacteriocin promoter regions**

The bacteriocin clusters associated with bactofencin A, salivaricin P, T and ABP-118 were examined to identify promoter sequences preceding the genes encoding the bacteriocin structural peptides (Fig. 2). Specifically, regions were examined for typical sigma 70 motifs (-10 (TATAAT) and -35 sequences (TTGACA)) and direct repeats using manual annotation

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and promoter mining software. Directly upstream of the bactofencin A start codon was a predicted sigma 70 promoter with -10 and -35 motifs located 16 bp apart and 23 bp upstream from the likely RBS site (Fig. 2a). A fragment of 110 bp incorporating this intergenic region was amplified and fused directly to the *gfp* gene in the pNZ44 plasmid to make *bfnA*prom.*gfp* (Table 2).

Analysis of the regions upstream of the prepeptide and structural genes in the class IIb bacteriocins did not reveal correspondingly obvious promoter regions. Alignment of the intergenic regions from the gene clusters highlight a very high sequence similarity (Fig. 2 b-d), with 98-99% nucleotide identity across the putative promoter regions for these bacteriocins and therefore, in certain cases, the same constructs were used to represent one putative promoter region (Table 2). In all, 4 regions, including (i) an ABP118-specific region of 345bp upstream of the ABP-118 prepeptide and (ii-iv) 3 promoter regions (*sal*prom1-3) ranging from 151bp-847bp representing 3 upstream regions of the structural genes of salivaricin P and salivaricin T, were amplified (Fig. 2b-d). *sal*prom1 represents a small putative promoter region (Fig S1) upstream of the bacteriocin prepeptide, whereas the larger regions (*sal*prom2 and *sal*prom3) were included to ensure cloning of promoter regions that may be located further upstream (Table 2).

Care was taken in the cloning of the larger regions to reduce the amount of translated nucleotides included in the final constructs. To create a bank of *gfp+* strains, the promoter.*gfp* fusions were transformed (in most cases; Table S2) into each of the 4 bacteriocin-producing strains; *L. salivarius* DPC6502 (bactofencin A producer), *L. salivarius* DPC6488 (salivaricin TL producer), *L. salivarius* DPC6189 (salivaricin P and bactofencin A producer) and *L. salivarius* NCIMB 40829 (**LSUCC118^{TM}**) (ABP-118 producer). In addition, a promoter-less construct containing the *gfp* gene was also made in each background to serve as a negative control (Table 2 and Table S2).

***In vitro* analysis the promoter region of the class IId bacteriocin bactofencin A**

The strength of the putative promoter regions from the bacteriocin clusters were assessed using GFP as a reporter. Initially each bacteriocin promoter was analysed in the natural background where the corresponding bacteriocin is produced. A comparison of fluorescence was performed by visualising stationary phase cells under a fluorescence microscope and followed by a comparison of expression monitored over 20 h of growth (Fig. S2). Cloning of the promoter region *bfnA*prom highlighted strong promoter activity initially in the *L. salivarius* DPC6502 (bactofencin A-producing) background, which increased further during the logarithmic growth phase (Fig. 3a). It should be noted here that T0 RFU readings are higher than expected; this is possibly due to an initial adjustment of the instrument.

Analysis revealed also that although the bactofencin A promoter is switched on in all 4 backgrounds tested, GFP expression was at a lower level in the backgrounds where bactofencin A is naturally produced, i.e. *L. salivarius* DPC6502 and *L. salivarius* DPC6189, than in the backgrounds where the bacteriocin is not encoded, i.e. *L. salivarius* NCIMB 40829 (**LSUCC118^{TM}**) and *L. salivarius* DPC6488. This was evident during growth analysis in log phase (Fig. 3b) and also when washed cells were viewed under a fluorescence microscope (Fig. 3c).

***In vitro* analysis of the promoter regions of the structural genes of class IIb bacteriocins**

The predicted promoter regions (*sal*prom1-3 and *abp-118*prom) representing salivaricin P, salivaricin T and ABP-118 respectively were expressed as GFP fusions in each of salivaricin-producing backgrounds to analyse promoter activity (Table S2). In all instances, these promoters exhibited weak expression during growth analysis in MRS broth (represented by *sal*prom2 in Fig. 4). Indeed, relative fluorescence units (RFU) values resembled those of the

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negative (promoter-less GFP) control (Fig. 4). An increase in promoter activity was not observed with either *sal*prom1 or *sal*prom3 in the *L. salivarius* DPC6189 background (not shown). It was thought that this may be due to an insufficient level of IP production and so the predicted inducing peptide for salivaricin P and for the salivaricin T/ABP-118 (genes corresponding to the IPs for salivaricin T and ABP-118 show 100 % nucleotide identity) were synthesised and added at varying concentrations and fluorescence was monitored over 20h. The addition of 10^{-5} M of the salivaricinT/ABP-118 IP to cells expressing the promoter region *sal*prom2 and *abp-118*prom resulted in an increase in fluorescence over time (represented by *sal*prom2 in Fig. 4). The use of the ABP-118 IP to induce bacteriocin activity in *L. salivarius* NCIMB 40829 (**LSUCC118^{TM}**) has been previously reported (11).

Bacteriocin promoter activity under simulated environmental conditions

A number of challenge assays were undertaken to establish if environmental signals, and in particular those associated with the GIT, could induce bacteriocin promoter activity with a view to determining if the bacteriocins might still be produced under the stressful conditions present in the gut or if information could be gained that would facilitate enhanced bacteriocin production in a processing environment. Cells containing the promoter.*gfp* fusions were challenged and analysed for promoter activity at sub-inhibitory growth levels of low pH, salt, bile, in the presence of target microbes and at low levels of simulated gastric juice. For each challenge assay, specific promoter fusions were analysed in their own bacteriocin-producing backgrounds. The bactofencin A promoter was functional under all conditions tested including at examples of low pH [5.5], in the presence of bile [0.2% (w/v)] or in the presence of target microbes, with GFP expression comparable to that detected in the *L. salivarius* DPC6502 strain in lab media (data not shown). In the presence of low levels of added salt [0.25-0.5% (w/v)], promoter activity actually increased and had higher GFP levels than

controls (Fig 5a). Cells were also exposed for short times (30 secs, 1 min and 5 mins) to gastric fluid [pH2], mimicking the conditions of the upper intestinal tract. Under these stressful conditions, GFP expression increased, indicating induction of *bfnA*prom when exposed to the harsh conditions such as those encountered in the gut (Fig 5b).

The class IIb bacteriocin promoter regions were also tested in response to environmental stresses described above, however no significant increase in promoter activity was observed (data not shown).

DISCUSSION

Bacteriocins have great potential as therapeutic agents that can be used to inhibit important pathogens in food and/or clinical settings. The level at which bacteriocins are produced under stressful environmental conditions is not an area that has been extensively explored. In this study, we used a stable expression system for monitoring the activity of *L. salivarius*-associated bacteriocin promoters under various environmental conditions.

This study initially analysed 3 different potential reporter systems to study promoter activity in *L. salivarius* cells. The GFP reporter was found to be more optimal in the bacteriocin-producing backgrounds than either the RFP or Lux representatives. Interestingly, a very recent study has reported the development of an optimised GFP variant suitable for anaerobic environments (39) as the strains expressing GFP in this study were analysed under microaerobic conditions to facilitate fluorescence detection. Both the RFP and Lux systems were also investigated due to their usefulness in live tissues. However, neither system facilitated efficient reporter expression in *L. salivarius*. This is in accordance with previous work on the Lux system whereby it was suggested that the *L. salivarius* genome does not contain the genetic machinery to support the light emitting reaction. The authors suggested that *L. salivarius* lacks a NAD(P)H:FMN oxidoreductase, which functions to synthesise

reduced flavin mononucleotide (FMNH2) required to emit bioluminescence in the presence of oxygen (38). There are limited studies on the use of the DsRed (RFP) protein in *Lactobacillus* spp. but there have been some reports of RFP expression in *Lactobacillus* cells (40). In this work the expression of the DsRed (RFP) protein was initially optimised for expression in *E. coli* but was not efficiently expressed in *L. salivarius*. This may be due to weak RBS initiation in the *Lactobacillus* background but further analysis would be required to determine this.

Assessment of the class IId bacteriocin bactofencin A promoter.*gfp* fusions revealed a strong promoter active under all environmental stresses, the activity of which could be increased under specific conditions and when introduced into non-bactofencin A producing strains (Fig. 3). It may be that, as for some other bacteriocin clusters, the associated promoter is switched on at a basal level in all conditions but once the bacteriocin peptide reaches a certain threshold, promoter activity is switched off or repressed (Fig. S3). The genetic determinants for such a bactofencin A-associated regulatory pathway have yet to be identified but, now that the existence of such a mechanism has been indicated, it will be the focus of further studies. In addition, the increased promoter activity upon exposure to mild stresses such as low levels of salt and simulated gastric fluid (Fig. 5) that may mimic the conditions of the GIT suggest the potential to use this bacteriocin to inhibit gut pathogens *in vivo*. Given that gastric fluid contains salt, we cannot preclude the possibility that salt also plays a key role in promoter induction in this environment.

Analysis of the genomic regions upstream of the structural genes of the 3 class IIb bacteriocins, salivaricin P, salivaricin T and ABP-118, did not reveal obvious promoter regions. However, these promoter regions are likely to be bound by a response regulator from a two-component system and therefore may not contain typical sigma 70 promoter motifs. In addition, it should be noted that in low GC organisms, stretches resembling – 10 elements can

be frequent and therefore definitive identification of promoter elements can be challenging. The *in vitro* assays investigating the promoters from the class IIb bacteriocins revealed apparent weak expression. Given that the addition of the IP stimulated promoter activity (Fig. 4), it is likely that the full promoter region is indeed cloned and active but sensitive to the levels of IP available.

In conclusion, this study investigated the activity of the promoters of bacteriocin production in *L. salivarius*. The results highlight the capacity for salt (bactofencin A) or an induction peptide (class IIb salivaricins) to induce promoter activity, information that can be taken advantage of to increase bacteriocin production in the GIT or in the processing environment.

Figure Legends

Fig. 1 Schematic representation of the pNZ-based fluorescent plasmids constructed for the expression of *gfp*, *rfp* and *lux* genes in *E. coli* and *L. salivarius* cells.

Fig. 2 *In silico* analysis of the bactofencin A putative promoter region upstream of the bactofencin structural gene (a). Alignment of the structural genes, immunity genes and the intergenic regions of the class IIb bacteriocins salivaricin P (b), ABP-118 (c) and salivaricin TL (d).

Red arrows; bacteriocin structural genes, orange arrows; genes encoding bacteriocin prepeptide, green arrows; regulatory genes/transport genes, white arrow; genes encoding bacteriocin inducing peptides, purple arrow; bacteriocin immunity genes. The green blocks

between clusters represent regions of high % nucleotide identity from within the promoter regions were amplified. The grey blocks represent regions of high % nucleotide identity in the surrounding genes. The values of % nucleotide identity are included within the panels.

Fig. 3 Production of total fluorescence (as measured by RFU) produced by the *bfnA*prom.*gfp* plasmid in the bactofencin A producer *L. salivarius* DPC6502 (green bar chart) and growth analysis of the *L. salivarius* DPC6502 strain over time as measured by OD 600nm (a). Production of total fluorescence produced by the *bfnA*prom.*gfp* plasmid in bacteriocin producers *L. salivarius* **UCC118**; ◯, DPC6502; ◆, DPC6189; ▲, DPC6488; ×.

The underneath panel shows *L. salivarius* DPC6189 (BfnA +ve) and *L. salivarius* DPC6488 (BfnA −ve) expressing *bfnA*prom.GFP cultures under a fluorescence microscope (c).

Fig. 4 Production of total fluorescence (as measured by RFU) produced by the *sal*prom2.*gfp* in *L. salivarius* **UCC118** in MRS broth and in response to 10^{-5} M ABP-118/salT IP.

gfp negative control; ■ , *sal*prom2 in MRS broth;● , *sal*prom2 in MRS broth with IP ◯.

Fig. 5 Production of total fluorescence (as measured by RFU) produced by *bfnA*prom.*gfp* in *L. salivarius* DPC6502 in response to salt (a) and to simulated GI fluid (b). *bfnA*prom.*gfp* in MRS broth;◆, *bfnA*prom.*gfp* in MRS broth with 0.5% w/v NaCl; ◊, *bfnA*prom.*gfp* in MRS broth following 5 min exposure to simulated GI fluid;◈ .

Table 1. Strains and plasmids used in this study

Strain or Plasmid	Relevant Properties	Source or Reference
Strains		
E. coli		
DH10B	F- *mcrA* Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ *lac*X74 *rec*A1 *ara*D139 Δ(*araleu*)7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G. Host for pNZ44 fluorescent derivatives.	Life Technologies
L. salivarius		
UCC118	Human ileocecal isolate, ABP-118 bacteriocin producer	(11)
DPC 6488	Human isolate, Salivaricin TL producer	(8)
DPC 6502	Porcine isolate, Bactofencin A producer	(9)
DPC 6189	Porcine isolate, Salivaricin P and Bactofencin A producer	(8, 13)
L. delbrueckii subsp. *bulgaricus* LMG 6901	Bacteriocin sensitive indicator	(13)
Plasmids		
pNZ44	Cm^r, Lactococcal expression vector	(41)
pEVS*gfp+*	source of *gfp* gene	(28)
pDsRed express vector	Amp^r, lacZ-DsRed-Express fusion protein	Clontech
		(30)
pPl2Lux	Derivative of the listerial integration vector pPL2 and harbors the synthetic luxABCDE	

Cm^r: chloramphenicol resistance; Amp^r: Ampicillin resistance

Table 2: Fluorescence plasmids constructed in this study

Plasmid	Relevant Properties
pNZ44.*gfp*	pNZ44 plasmid with the *gfp* gene fused to the p44 promoter.
pNZ.gfp-	promoter less pNZ44 derived plasmid containing the intact *gfp* gene.
pNZ44.*rfp*	pNZ44 plasmid with the *rfp* gene fused to the p44 promoter.
pNZ44.*lux*	pNZ44 plasmid containing the *lux*ABCDE genes fused to the p44 promoter.
*cysk*prom.*gfp*	pNZ44 plasmid containing the *gfp* gene, the p44 promoter is replaced with the promoter of the *cysK* gene of *L. salivarius* **UCC118**
*bfnA*prom.*gfp*	pNZ44 plasmid containing the *gfp* gene, the p44 promoter is replaced with the putative promoter of the bactofencin A structural gene of *L. salivarius* DPC6502
*abp-118*prom.*gfp*	pNZ44 plasmid containing the *gfp* gene, the p44 promoter replaced with the putative promoter region upstream of the ABP-118 prepeptide of *L. salivarius* **UCC118**
*sal*prom1.*gfp*	pNZ44 plasmid containing the *gfp* gene, the p44 promoter is replaced with an 847bp region upstream of the salivaricin P and T structural genes *of L. salivarius* DPC6189 and DPC6488 respectively. (Oligonucleotides used include *sal*prom1For and *sal*prom2Rev in which cloned insert begins 847 bp upstream of the start codon of the structural gene (*sal*T α of DPC6488 and *sln*1 of DPC6005).
*sal*prom2.*gfp*	pNZ44 plasmid containing the *gfp* gene, the p44 promoter is replaced with a 391bp region upstream of the salivaricin P and T structural genes of *L. salivarius* DPC6189 and DPC6488 respectively. (Oligonucleotides used include *sal*prom2For and *sal*prom2Rev in which cloned insert begins 391 bp upstream of the start codon of the strucutural gene *sal*T α of DPC6488 and *sln*1 of DPC6005).
*sal*prom3.*gfp*	pNZ44 plasmid containing the *gfp* gene, the p44 promoter is replaced with the synthesised 151bp region (sequence in Fig S1) upstream of the salivaricin P and T prepeptide of *L. salivarius* DPC6189 and DPC6488 respectively.

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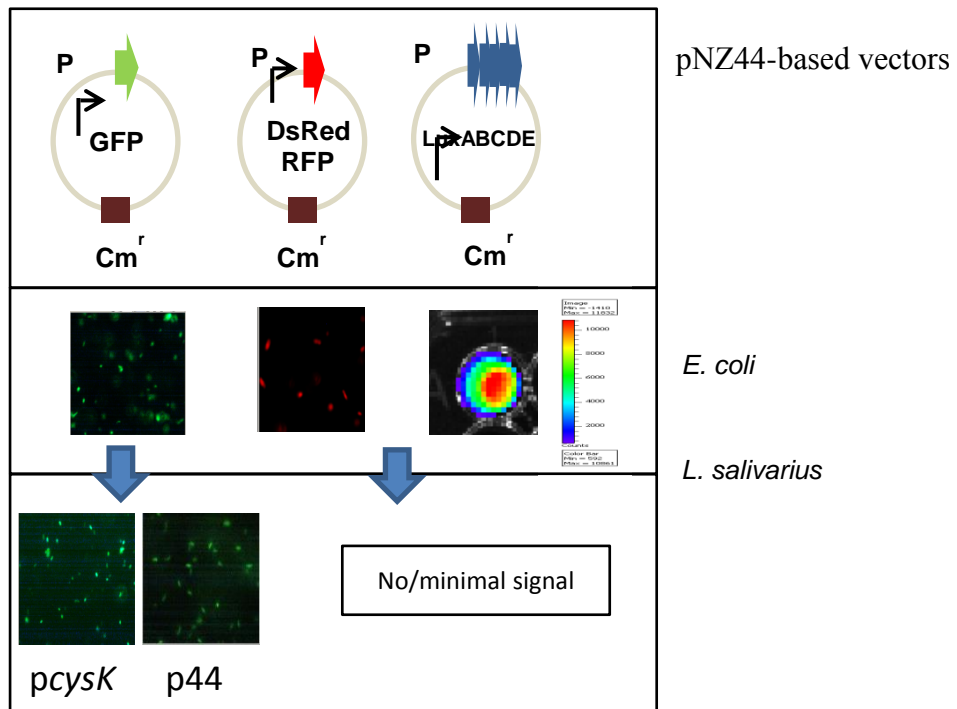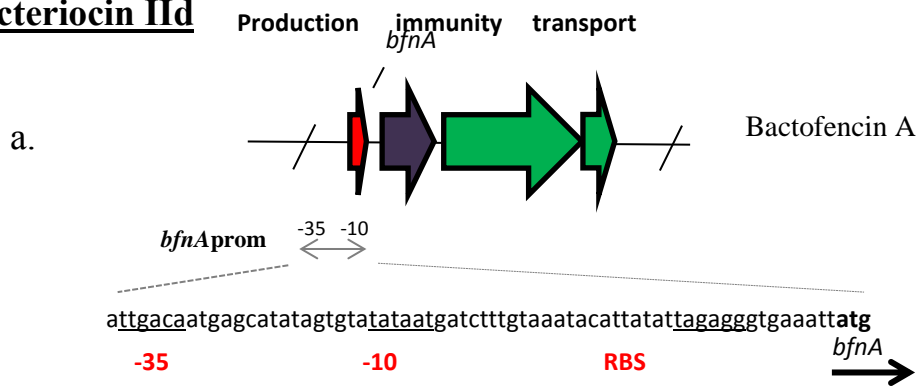
Fig. 1

pNZ44-based vectors

E. coli

L. salivarius

No/minimal signal

p*cysK*   p44

Fig. 2

Bacteriocin IId

Production | immunity | transport
bfnA

a.   Bactofencin A

*bfnA*prom   -35 -10

attgacaatgagcatatagtgtatataatgatctttgtaaatacattatattagagggtgaaattatg

-35   **-10**   **RBS**

bfnA

Bacteriocin IIb

*sal*prom1
*sal*prom2
*sal*prom3   *sln1 sln2*

b.   Salivaricin P

85% 92% 93%   97%

abp118α   *abp118β*

abp-118prom   *abpIM   abpIP   abpK   abpR*

c.   ABP-118

96%

salTα   *salTβ*

d.   Salivaricin TL

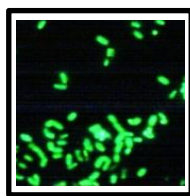**Production/   Regulation**
immunity

Fig. 3
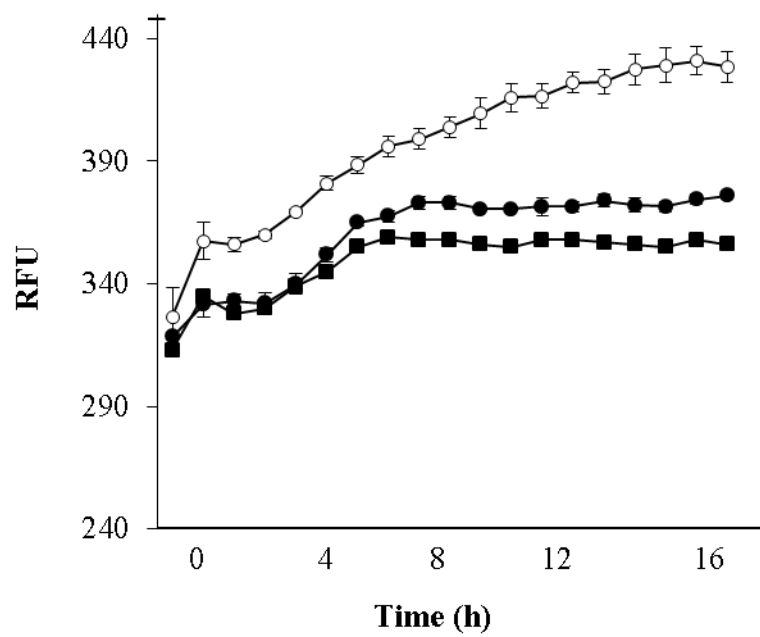
a.

b.

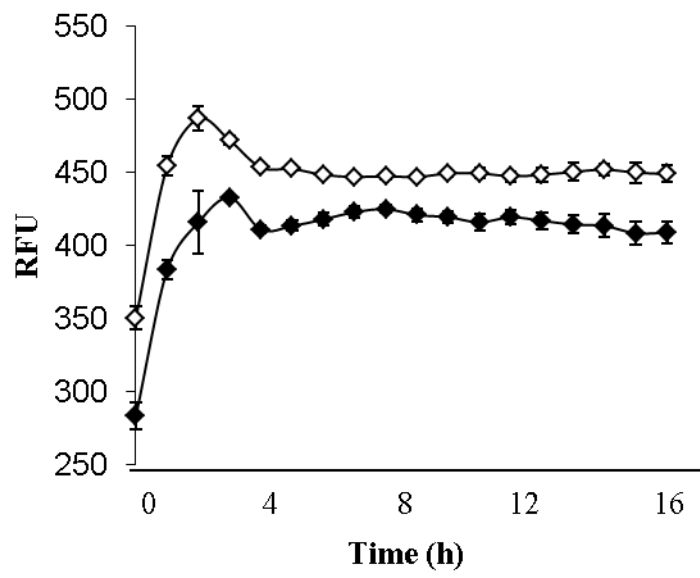

c.

BfnA +ve (DPC6189)   BfnA −ve (DPC6488)

Fig. 4

Fig. 5

a.

b.