Production of multiple bacteriocins from a single locus by gastrointestinal strains of *Lactobacillus salivarius*

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Abstract

Bacteriocins produced by *Lactobacillus salivarius* isolates derived from gastrointestinal origin have previously demonstrated efficacy for *in vivo* protection against *Listeria monocytogenes* infection. In this study, comparative genomic analysis was employed to investigate the intraspecies diversity of seven *L. salivarius* isolates of human and porcine intestinal origin, based on the genome of the well characterised bacteriocin-producing strain *L. salivarius* UCC118. This revealed a highly conserved megaplasmid-encoded gene cluster in these strains involved in the regulation and secretion of two-component class IIb bacteriocins. However, considerable intraspecific variation was observed in the structural genes encoding the bacteriocin peptides. These ranged from close relatives of abp118 such as salivaricin P, which differs by 2 amino acids, to completely novel bacteriocins such as salivaricin T, which is characterized in this study. Salivaricin T inhibits closely related lactobacilli and bears little homology to previously characterized salivaricins. Interestingly, the two peptides responsible for salivaricin T activity, SalTα and SalTβ, share considerable identity with the component peptides of thermophilin 13, a bacteriocin produced by *Streptococcus thermophilus*. Furthermore, the salivaricin locus of strain DPC6488 also encodes an additional novel one-component class IId anti-listerial bacteriocin, salivaricin L. These findings suggest a high level of redundancy in the bacteriocins that can be produced by intestinal *L. salivarius* isolates using the same enzymatic production and export machinery. Such diversity may contribute to their ability to dominate and compete within the complex microbiota of the mammalian gut.

Introduction

There is increasing evidence to suggest that bacteriocin production is a desirable probiotic trait which enables the establishment and persistence of the producing strains within the gastrointestinal tract (GIT) (2, 9, 15, 35). These antimicrobials may have a narrow or broad spectrum of inhibition (16). Although broad spectrum bacteriocins, such as nisin, have been useful with respect to the control of spoilage and pathogenic organisms in food preservation (8), there has been increasing interest in narrow spectrum bacteriocins, such as thuricin CD, due to their narrow specificity and minimal impact on non-target beneficial GIT microbes (30). As such, these bacteriocins offer potential alternatives to traditional antibiotics with respect to controlling pathogens within the gut (29). *In situ* production of such narrow spectrum bacteriocins by probiotics would overcome complications such as the proteolytic degradation of orally delivered antimicrobial peptides during gastric transit. *Lactobacillus salivarius* is a promising probiotic candidate frequently isolated from human, porcine and avian GITs, many of which are producers of unmodified bacteriocins of class IIa (pediocin-like bacteriocins), class IIb (two-component bacteriocins) and class IId (linear non-pediocin-like bacteriocins) (4, 13, 31). Significantly, an *in vivo* demonstration of the anti-infective properties of the abp118-producing strain *L. salivarius* UCC118 has established the *in vivo* functionality of such bacteriocins (6). In addition, purified OR7, a class IIa bacteriocin produced by the chicken intestinal isolate *L. salivarius* NRRL B-30514, has also been successfully employed to reduce *Campylobacter jejuni* colonization in poultry (31).

Closely related variants of abp118 (a two-component class IIb bacteriocin) frequently occur in intestinally-derived *L. salivarius* from different hosts (2, 17, 26, 27), suggesting that this feature may be important for the successful establishment of

L. salivarius within the GIT. Further evidence of the ecological advantage that this trait bestows upon the producing strains was provided by the salivaricin P-producing *L. salivarius* DPC6005, which prevailed over four components of a probiotic formulation within the porcine ileum (34). The production of analogous bacteriocins by genetically-distinct strains and species is frequently ascribed to extensive horizontal gene transfer events occurring within the GIT (1, 2, 15, 22, 33). It is thus notable that abp118 is encoded on a megaplasmid in *L. salivarius* UCC118. Although the transfer-associated genes within the conjugative megaplasmid pMP118 appear non-functional in *L. salivarius* UCC118 (10), corresponding *RepA*-type megaplasmids are universally present in *L. salivarius* (17). Interestingly, Wescombe *et al*., (2006) demonstrated the *in vivo* conjugative transmission of bacteriocin-rich megaplasmids in *Streptococcus salivarius* species, which was associated with the numerical prominence of the species within the oral cavity (35).

The variable nature of *L. salivarius* with respect to their ability to produce bacteriocins, and the nature of the bacteriocins which they produce, is becoming increasingly apparent. It has been noted that many *L. salivarius* strains which harbour homologues of the abp118 structural genes do not display anti-*Listeria* activity (17). A recent comprehensive genomic analysis revealed that the three-component regulatory system responsible for the transcriptional regulation of abp118 production was not well conserved and was likely responsible for the bacteriocin negative phenotype of these strains (28). Other strains produce closely related, yet distinct, bacteriocins such as salivaricin P which differs from abp118 with respect to two amino acids in their respective β peptides (2). Interestingly, the nine associated polymorphisms (three in the α gene, six in the β gene) appear to be specific features associated with *L. salivarius* isolates derived from porcine intestinal origin (2). It

would thus seem that gene acquisition, mutation and/or gene decay, all of which are potential consequences of species adaptation to a specific ecological niche (3, 24), have impacted on the adaptation of this widespread bacteriocin locus of *L. salivarius*, driving genome-wide specialisation in response to a particular niche.

We conducted a genome-wide comparison of seven genetically distinct bacteriocin-producing intestinal *L. salivarius* strains isolated in our laboratory which revealed that the salivaricin/abp118-associated bacteriocin locus is a site of high variability. In the case of the neonatal isolate *L. salivarius* DPC6488, two novel narrow spectrum bacteriocins, salivaricin T, which displays considerable homology to a two-component class IIb bacteriocin associated with *Streptococcus thermophilus,* and salivaricin L, a one component class IId bacteriocin exhibiting anti-*Listeria* activity, have been characterised in this study.

Materials and Methods

Bacterial strains and culture conditions. *L. salivarius* strains of both human and porcine intestinal origin, previously isolated and characterised in our laboratory, were included in this study (Table 1). Lactobacilli were routinely cultured under anaerobic conditions at 37°C in MRS medium (Difco Laboratories, Detriot, MI). Anaerobic conditions were maintained with the use of anaerobic jars and Anaerocult A gas packs (Merck, Darmstadt, Germany). *Listeria innocua* DPC3572 and *Listeria monocytogenes* NCTC 11994 were grown aerobically at 37°C in BHI (Merck).

Pulsed-field gel electrophoresis analysis. Differentiation of the *L. salivarius* isolates was confirmed by PFGE as previously described (23). The megaplasmid content of the strains was also determined by PFGE following S1 nuclease treatment of high-molecular-weight DNA, also previously described (17).

DNA amplification, sequencing and analysis. Template DNA was extracted from *L. salivarius* DPC6005 and *L. salivarius* DPC6488 to amplify the genetic loci responsible for salivaricin production in the corresponding strains using primers designed specific to the locus of abp118 (accession number AF408405). A series of abp118-specific primers were used to amplify the salivaricin P locus of strain DPC6005 by routine PCR generating a contiguous sequence of approximately 13 kb. The primer pair 5’ CCGCCGATATACTATTCGTGG 3’ and 5’ GAGAGTTAGACCTGATGAAG 3’ was also used to amplify the 13 kb salivaricin P gene cluster in its entirety using Extensor Hi-Fidelity PCR mastermix (Abgene, Surrey, UK) under conditions recommended by the manufacturer.

A PCR amplicon previously generated using DPC6488 template DNA and primers specific for the amplification of the salivaricin P structural genes (23) generated approximately 900 bp sequence data in the present study. Following sequence analysis, the abp118-specific primer pair above was also employed to amplify the 12 kb salivaricin gene cluster of strain DPC6488. Oligonucleotide primers were synthesised by Sigma-Genosys (Poole, Dorset, UK) and purified amplicons were sequenced by Beckman Coulter Genomics (Essex, UK). Alignments and analyses of sequence data were performed using LASERGENE 6 software (DNAStar Inc., Madison, WI). Database searches were performed using the basic local alignment search tool (BLAST) on the National Centre for Biotechnology information (NCBI) server (http://www.ncbi.nlm.nih.gov). Open reading frames (ORFs) were identified using LASERGENE 6, ORF finder and glimmer on the NCBI server and Genemark, gene prediction software (http://exon.biology.gatech.edu).

Microarray-based Comparative Genomic Hybridisation. Comparative genomic hybridizations were performed using a highly replicated custom microarray (Agilent Technologies, CA, USA) designed based on the genome of *L. salivarius* UCC118 as previously described (5, 11, 28). The experimental procedures for genomic DNA extraction, fragmentation and fluorescent labelling and co-hybridisation experiments of fluorescently-labelled gDNA of the test and reference strains employed in this study were recently described (28). Two-dye-swap replicate hybridisations were performed for each strain tested and self-hybridisation of the reference strain was carried out as a control experiment. Analysis of the microarray data were also as described by Raftis *et al*. (28). The sequencing data obtained for our strains was in agreement with the empirically determined cutoff intervals used to discriminate

between the presence, divergence and absence of genes previously selected on the basis of a comparison of the output data of a BLASTn comparison of the UCC118-specific oligonucleotide probe set with the sequence of the draft genome of the type strain *Lactobacillus salivarius* DSM20555 (accession number NZ_ACGT00000000) with the $\log_2$-transformed signal ratios determined for the hybridisation reaction of the gDNA of the corresponding strain (28). Therefore, these cuttoff intervals were also applied to the present dataset. Cutoff intervals of (≥ -1.5), (< -1.5, ≥ -2.4), (< -2.4, ≥ -4.5), (< -4.5, ≥ -5.8) and (< -5.8) corresponded to highly conserved, conserved, divergent, highly divergent, and absent features in the test strain relative to *L. salivarius* UCC118, respectively. Hierarchical clustering of strains was performed using the complete linkage clustering and results were visualised using Genesis software (32).

Purification of the antimicrobial peptides produced by *L. salivarius* DPC6488.

The antimicrobial peptides were purified from a 2 L overnight culture of *L. salivarius* DPC6488 grown in MRS media. The cells were harvested by centrifugation at 8,000 × g for 15 min, resuspended in 250 mL 70% (vol/vol) propan-2-ol containing 0.1% (vol/vol) trifluoric acid (TFA) and stirred at room temperature for 3 h. Cells were removed by centrifugation, and propan-2-ol by rotary evaporation. The resultant preparation was applied to a 5.0 g (20 mL volume) Strata C18 solid phase extraction (SPE) column (Phenomenex, Cheshire, UK) pre-equilibrated with methanol and water. The column was washed with 35% (vol/vol) ethanol and the antimicrobial peptides were subsequently eluted with 70% (vol/vol) propan-2-ol containing 0.1% (vol/vol) TFA. Following removal of the propan-2-ol from the preparation, 4 ml aliquots were applied to a Jupiter proteo C12 reversed phase-high pressure liquid

chromatography (RP-HPLC) column (250.0 × 10.0 mm, 4 µm particle size, 90 Å pore size; Phenomenex) pre-equilibrated with 25% (vol/vol) acetonitrile 0.1% (vol/vol) TFA. The column was developed in a gradient of 25% to 55% (vol/vol) acetonitrile containing 0.1% (vol/vol) TFA from 5 to 50 min at a flow rate of 2.5 mL min^{-1}. Individual fractions were assayed by well diffusion using the sensitive indicator strain *L. bulgaricus* LMG 6901, and matrix-assisted laser desorption ionisation-time of flight (MALDI-TOF) mass spectrometry (MS) was performed to determine fractions containing the peptides of interest. Fraction 27, containing a mass of 4433 Da corresponding to the salivaricin B peptide, was applied to a Luna analytical SCX cation exchange HPLC column (250.0 × 4.6 mm, 5 µm particle size, 100 Å pore size; Phenomenex), for further purification, following removal of acetonitrile by rotary evaporation. Using buffer A (20 mM potassium phosphate containing 25% (vol/vol) acetonitrile, pH 2.5*)*, and buffer B (20 mM potassium phosphate, 25% (vol/vol) acetonitrile, 1 M potassium chloride, pH 2.5) the column was pre-equilibrated with 10% buffer B and subsequently developed in a gradient of 10% to 65% buffer B, from 5 to 45 min at a flow rate of 1.0 ml min^{-1}. Individual fractions of interest were applied to a 200 mg (3 ml volume) Strata C18 SPE column (Phenomenex) pre-equilibrated with methanol and water. The column was washed with 30% (vol/vol) ethanol and 40% (vol/vol) propan-2-ol, followed by the elution of the antimicrobial component with 70% (vol/vol) propan-2-ol containing 0.1% (vol/vol) TFA. Similarly, fractions 38-40, containing masses of 5655 Da and 5267 Da corresponding to the respective SalTα and SalTβ peptides, were pooled, concentrated and applied to the Luna analytical SCX cation exchange HPLC column for separation and purification as described above, as were fractions 44-46, in which a mass of 4117 Da was detected corresponding to the mature salivaricin L peptide. Bacteriocin activity was monitored

throughout purification by well diffusion assay using the sensitive indicator strain *L. bulgaricus* LMG 6901. MALDI-TOF MS was performed as previously described (7).

Peptide synthesis. Synthetic analogues of mature salivaricin B and salivaricin L were synthesised according to the deduced amino acid sequence of *slnT3* and ORF13 (*slnL*) using microwave-assisted solid phase peptide synthesis (MW-SPPS) performed on a CEM Liberty^{TM} microwave peptide synthesiser using a H-Ser-HMPB-ChemMatrix® and a H-Cys(TRT)-HMPB-ChemMatrix® resin (PCAS Biomatrix Inc. Quebec, Canada), respectively. The molecular masses of the synthetic analogues were confirmed using MALDI TOF MS and the antimicrobial activity of the crude peptides was assayed by well diffusion. Synthetic salivaricin L was purified by RP-HPLC using a Jupiter C5 column (250.0 × 10.0 mm, 10 µm particle size, 300 Å pore size; Phenomenex) developed in a gradient from 25% to 55% (vol/vol) acetonitrile containing 0.1% TFA from 10 to 50 min at a flow rate of 2.5 mL min^{-1}. Fractions containing the desired molecular mass, identified by MALDI TOF MS, were pooled and lyophilised using a Genevac HT 4X lyophiliser (Genevac Ltd. Ipswitch, UK). The peptide was dissolved in 50% (vol/vol) acetonitrile at a concentration of 5 mg/ml and stored at -20°C under nitrogen. Appropriate dilutions of the peptides in 50mM potassium phosphate buffer pH 6.8 were used for bacteriocin assays.

Specific activity analysis of salivaricin L. A microtiter plate assay system was used to determine the minimum concentration of the synthetic salivaricin L analogue required to inhibit growth of the indicators, *L. innocua* DPC3572 and *L. monocytogenes* NCTC 11994 by 50% (MIC_{50}). The microtitre plate was first treated with bovine serum albumin (BSA) to prevent adherence of the peptide to the sides of

the wells, as described previously (12). Each plate included triplicate assays at each concentration examined. Each well contained a total volume of 200 µl, comprised of purified synthetic salivaricin L, 150 µl of a 1-in-10 dilution of the indicator culture (A_{590} of 0.5) in BHI broth. Control wells contained media only (blanks), or untreated indicator culture. The microtiter plate cultures were then incubated at 37°C and the optical density at 590 nm (OD_{590}) recorded at hourly intervals for 6 h (GENios plus; TECAN, Switzerland). Triplicate readings were averaged and blanks were subtracted from these readings. The amount of bacteriocin that inhibited the indicator strain by 50% was defined as 50% of the final OD_{590} ± 0.05 of the untreated control culture.

Results

The seven *L. salivarius* test strains included in this study were of either human or porcine intestinal origin (Table 1), and were specifically selected by their ability to produce bacteriocins which we propose constitutes an important probiotic trait (6, 31, 34). For comparative reasons, one isolate of human origin with a bacteriocin negative phenotype, DPC6196, was also included in the genotypic analysis as it harbours homologues of the abp118 bacteriocin structural genes (2). Due to the localisation of the abp118 bacteriocin gene cluster on the megaplasmid of strain UCC118 (17), the megaplasmid content of the test strains was first compared with that of UCC118.

Analysis of the megaplasmid content of bacteriocin-producing *L. salivarius* isolates. Pulsed field gel electrophoresis (PFGE) of total genomic DNA confirmed that the seven test strains were genetically distinct (data not shown), and the megaplasmid content of each, as determined by S1 PFGE, is outlined in Table 2. The human derived isolates DPC6196 and DPC6488 were found to harbour megaplasmids

of 180 and 195 kb, respectively. Smaller plasmids were also identified in these strains, two in DPC6488 of approximately 20 kb and 30 kb, and one in DPC6196 of approximately 48 kb. The porcine derived isolates, DPC6005, DPC6502 and 7.3, each contained megaplasmids of approximately 242 kb, similar to pMP118 (5), while DPC6027 and DPC6189 contained larger plasmids of 320 kb and 360 kb, respectively. Multiple megaplasmids were observed in *L. salivarius* DPC6189 and *L. salivarius* 7.3 which both appear to harbour an additional linear megaplasmid of approximately 195 kb. The linear nature of these megaplasmids was determined when, under different switching conditions, PFGE analyses revealed that the corresponding bands migrated to the same location on the gel regardless of whether DNA from these isolates was digested with S1 nuclease or not (data not shown).

Genomic diversity of *L. salivarius* strains. Array comparative genomic hybridisation (aCGH) analyses revealed considerable genomic diversity between strain UCC118, the five Bac^+ (bacteriocin-producing) porcine isolates and the human-derived Bac^+ strain DPC6488 and Bac^- (bacteriocin negative, despite harbouring homologues of the abp118 structural genes) strain DPC6196, consistent with results reported by Raftis *et al*., (2011) (28). We particularly noted a high level of plasticity within the UCC118 megaplasmid-associated bacteriocin abp118-encoding locus.

Genetic diversity of the salivaricin bacteriocin locus in *L. salivarius*. The genetic determinants responsible for the production of abp118 are comprised of the abp118 α and β structural genes, accompanied by the genes encoding a cognate immunity protein, a three-component regulatory system, an ABC-transporter and transport accessory protein which are responsible for cleavage and secretion of the mature

active bacteriocin (13) (Fig. 1). Six additional ORFs are also present on the 10.7 kb abp118 locus, three of which encode putative bacteriocin-like precursor peptides, two of which have no homologues while one was identified as a pre-salivaricin B homologue (4, 13). Each of the *L. salivarius* test isolates included in this study are bacteriocinogenic, with the exception of DPC6196, and sequence analysis revealed that the abp118 gene cluster shares greater than 90% sequence similarity with that of salivaricin P (data not shown (13)). Despite this, CGH revealed considerable genetic diversity with respect to the bacteriocin locus across the test strains (Fig. 2). The porcine intestinal strain DPC6502 was particularly notable by virtue of lacking abp118-related homologues, despite having an antimicrobial-producing phenotype (Fig. 2, (23)). This observation eliminates the possibility that this strain produces abp118, salivaricin P and/or salivaricin B. Although CGH data indicated that the bacteriocin structural genes, the genes encoding the response regulator and the transport system of abp118 were conserved in each of the remaining porcine derived isolates, DPC6005, DPC6027, DPC6189 and 7.3, the genes encoding the immunity, induction peptides and the histidine kinase component of the salivaricin regulatory system of these isolates were divergent from their respective abp118 counterparts, as illustrated in Fig. 2. Indeed, sequence analysis of the salivaricin P locus of the prototype producing strain DPC6005 (data not shown) confirmed this.

Overall, the abp118 locus was most highly conserved in the human isolate DPC6196 (Fig. 2). Although displaying a Bac^- phenotype, this isolate harbours homologues of *abp118α* and *abp118β*, differing by just 1 and 2 nucleotides, respectively (2). CGH analysis revealed divergence with respect to the *abpT* (LSL_1910) and *abpD* (LSL_1909) homologues, required for secretion of the mature active bacteriocin, in DPC6196 which is likely responsible for the Bac^- phenotype of

this strain. Previously, PCR analysis using primers specific for the salivaricin P structural genes indicated the presence of related genes in *L. salivarius* DPC6488 (23). However, the unique genetic variability pattern of this neonatal isolate instead indicated that the genes corresponding to *abp118α* (LSL_1917) and *abp118β* (LSL_1916) were divergent and absent, respectively (Fig. 2). In contrast, the genes encoding the abp118 regulatory and transport systems were perfectly conserved in this strain, relative to UCC118, as was the gene encoding pre-salivaricin B (LSL_1921). As this strain is of human origin, an important consideration in the selection of commercial probiotics, the antimicrobial activity and corresponding genetic determinants of this isolate were further investigated.

Analysis of the gene products encoded by the salivaricin locus of *L. salivarius* DPC6488. A single primer pair successfully amplified the individual salivaricin loci of *L. salivarius* UCC118, DPC6005 and DPC6488 in their entirety. Sequencing of the corresponding amplicon of *L. salivarius* DPC6488 allowed for elucidation of the most divergent region of the bacteriocin locus of this strain, which on the basis of CGH data corresponds to the sequence between LSL_1913 and LSL_1924 of strain UCC118 (Fig. 2), and confirmed the conservation of the regulatory and transport systems of abp118 in this strain. *In silico* analysis of the sequence data resulted in the identification of 20 ORFs (Fig. 1; Table 3), five of which, ORF3-5, 7 and 13, encoded bacteriocin-like prepeptides. The putative products of ORF3 and ORF4 (correspondingly designated *slnT*3 and *slnT*4) were 98% and 97% identical to the salivaricin B precursor peptide (LSL_1921) and a bacteriocin-like prepeptide (LSL_1918) of UCC118, respectively. The deduced products of ORF5 and ORF7 also resembled bacteriocin precursor peptides with double-glycine leader sequences, and

were designated *slnTα* and *slnTβ* (Fig. 3A). The first 57 nucleotides of *slnTα* closely resemble those encoding the leader peptide of Abp118α and Sln1, differing by two nucleotides (and, in turn, one aa) (Fig. 3B). However, no significant homology was observed between the corresponding propeptide (61 aa) and Abp118α (45 aa). Indeed a BLAST search revealed that this peptide instead shared 47% identity with ThmA, one component of the two-peptide bacteriocin thermophilin13 produced by *Streptococcus thermophilus* (20) (Fig. 3B). The bacteriocin-like precursor peptide encoded by *slnTβ* displayed similarity to acidocin LF221 A, one peptide of a putative two-component bacteriocin produced by *Lactobacillus gasseri* (18) (Fig. 3B). Only partial sequence of the complementary peptide of LF221 A (37 C-terminal aa) was available for comparison with the propeptide encoded by *slnTα*, with which it shared 30% similarity (Fig. 3B). While the leader sequence of SalTβ also shares 42% similarity with that of Abp118α, no significant similarity was observed between the leader sequence of SalTβ and that of Abp118β (Fig. 3B). Indeed, the SalTβ leader sequence was most similar (59%) to that of the LafX peptide of lacticin F, produced by *Lactobacillus johnsonii* (Fig. 3B, (14)), while, perhaps most notably, the propeptide encoded by *slnTβ* shares 43% similarity with ThmB, the complementary peptide of ThmA (20).

Further *in silico* analysis of this cluster identified two ORFs encoding putative immunity proteins downstream of the potential bacteriocin structural genes *slnTα* and *slnTβ* (Fig. 3A). The deduced protein sequences corresponding to *slnT IM1* (ORF6) and *slnT IM2* (ORF8) displayed 81% and 76% identity with Abp118 IM. Despite being present in two copies, these LSL_1915-homologues were not detected by CGH due to the divergence in the nucleotide sequences of the genes in DPC6488 (72% and 69% identity, respectively, with *abpIM*). Three ORFs downstream of the putative

363 bacteriocin immunity gene, ORFs 9, 10 and 11, encode a putative induction peptide, a
364 sensory transduction histidine kinase and a response regulator, which shared 100%,
365 99% and 94% identity with their respective abp118 counterparts, thus confirming
366 conservation of the regulatory system of abp118 in strain DPC6488. The deduced
367 products of ORFs 12 and 14 did not share homology with any previously known
368 proteins. The 59 aa putative bacteriocin prepeptide encoded by ORF 13 (*slnL*) consists
369 of a double-glycine leader sequence of 18 aa and a mature peptide of 41 aa designated
370 salivaricin L, and shared greatest homology (59% identity) with a putative bacteriocin
371 precursor of the *Streptococcus* sp. C150 genome (accession no. EFX55741). This
372 putative bacteriocin precursor also shared very weak homology (38% and 37%) with
373 the precursor peptides of cerein 7B produced by *Bacillus cereus* Bc7 (25) and sakacin
374 Q produced by *Lactobacillus sakei* (21), respectively. Interestingly, both cerein 7B
375 and sakacin Q are single peptide class IId bacteriocins which are simultaneously
376 produced with cerein 7A and sakacin P by their respective producing strains (21, 25).
377 The deduced protein products of ORFs 15 and 16 share 99% and 97% identity with
378 those involved in the transport of abp118, AbpT and AbpD, respectively, thus
379 confirming conservation of the abp118 transport system in strain DPC6488. Three
380 ORFs downstream of the putative bacteriocin transport genes encoded proteins
381 homologous to the IS1223 family transposases. The protein products of overlapping
382 ORFs 17 and 18 shared 97% and 94% identity with the 61 C-terminal aa and 246 N-
383 terminal aa of transposase ISLasa2b encoded by LSL_1957, approximately 30 kb
384 downstream of the abp118 bacteriocin locus on pMP118. In addition, the deduced
385 product of ORF 19 shares 98% identity with transposase ISLasa1b encoded by
386 LSL_1958 of *L. salivarius* UCC118, indicative of a possible recombination event.
387 The final ORF identified on the salivaricin locus of DPC6488 encoded a hypothetical

protein of 274 aa, the C- and N-terminus of which shared 97% and 87% identity with the smaller hypotetical proteins encoded by LSL_1907 (174 aa) and LSL_1908 (76 aa) of the abp118 locus, respectively.

Isolation and characterisation of the bacteriocin-like peptides produced by *L. salivarius* DPC6488. The spectrum of activity of *L. salivarius* DPC6488, as determined by well diffusion assay of the neutralised cell free supernatant (CFS), was previously found to be limited to 12 closely related species of lactic acid bacteria (including *Enterococcus*, *Lactobacillus* and *Streptococcus* sp.) of a total of 62 indicator strains investigated. This activity was lost upon protease treatment with proteinase K, α-chymotrypsin, trypsin or pepsin (23). Cross sensitivity assays using the CFS of DPC6488 and of the abp118 and salivaricin P producers (UCC118 and DPC6005, respectively) revealed that all 3 strains are immune to both abp118 and the DPC6488-associated antimicrobials. Interestingly, however, both human isolates were sensitive to the CFS of the salivaricin P producer DPC6005, despite the fact that their respective immunity peptides share greater than 76% sequence identity.

Further investigations were performed to establish the nature of the antimicrobials produced by DPC6488. Mass spectral analysis revealed that peptides of mass corresponding to those of the mature products of *slnT3*, *slnTα*, *slnTβ*, and *slnL* were present in the culture supernatant of DPC6488. To determine if these peptides were responsible for the antimicrobial activity of the strain, the peptides were separated and purified using cation exchange chromatography. MS data confirmed the predicted molecular masses of the mature peptides encoded by *slnTα*, *slnTβ*, and *slnL*, 5,655 Da, 5,269 Da, and 4,117 Da, respectively, in the individually eluted active fractions. The individual SalTα and SalTβ peptides exhibited antimicrobial activity

against the sensitive indicator strain *L. delbrueckii ssp. bulgaricus* LMG 6901, which was further enhanced when they were combined (Fig. 4). Thus *slnTα* and *slnTβ* appear to encode a two-peptide bacteriocin that most closely resembles thermophilin 13, which we designated salivaricin T.

MS analysis also established that the mature salivaricin B analogue encoded by *salT3* is secreted by strain DPC6488 and identified the fraction containing a peptide of corresponding mass (4,433 Da). However, this peptide did not inhibit the *L. delbreueckii* ssp. *bulgaricus* indicator strain, nor did it display the anti-*Listeria* activity previously attributed to salivaricin B (4) (data not shown). As noted above, a gene encoding a homologue of the salivaricin B precursor peptide is also located on the abp118 gene cluster (LSL_1921) but was designated non-functional in *L. salivarius* UCC118 (5, 13). Indeed, CGH demonstrates that this gene is conserved in six of the seven test strains employed in this study and DNA sequence and MS data confirmed that the peptide encoded by *slnT3* of DPC6488 shares 100% identity with salivaricin B. To investigate further, a synthetic analogue of mature salivaricin B was generated based on the deduced aa sequence of *slnT3*. However, we were again unable to detect antimicrobial activity from the synthetic peptide (data not shown). Furthermore when either the synthetic analogue or the purified salivaricin B-containing fraction were combined with those containing SalTα and SalTβ, or the individual purified component peptides of salivaricin P, Sln1 and Sln2, salivaricin B failed to enhance their antimicrobial activity against *L. delbrueckii ssp. bulgaricus* LMG 6901 or *Listeria innocua* (data not shown). These findings thus question the previously reported anti-*Listeria* potency of this bacteriocin (4).

Interestingly, production of the fourth bacteriocin-like prepeptide encoded on the salivaricin gene cluster of strain DPC6488, with similarity to one-peptide class IId

bacteriocins, was also confirmed by MALDI-TOF MS. While this peptide inhibited three of the 12 indicator strains sensitive to salivaricin T, *L. delbrueckii ssp. bulgaricus, L. delbrueckii ssp. lactis* and *L. ruminis*, synergistic activity was not observed when combined with the salivaricin T component peptides SalTα and SalTβ. Moreover, the purified salivaricin L peptide also exhibited anti-*Listeria* activity. A synthetic analogue of the mature 41 aa peptide displaying similar activity to the natural peptide was employed to determine the specific activity of the bacteriocin, revealing an MIC_{50} of 20 µM for both *L. innocua* DPC3572 and *L. monocytogenes* NCTC 11994 (Fig. 5).

Given the inactivity of salivaricin B, and the antimicrobial activity of purified SalTα, SalTβ and salivaricin L, it is apparent that the inhibition of *L. delbrueckii ssp. bulgaricus* LMG 6901 strain by *L. salivarius* DPC 6488 can be attributed to two novel bacteriocins encoded within a single gene cluster, which we designate salivaricin T, corresponding to the two component peptide products encoded by *slnT*α and *slnT*β, and salivaricin L, corresponding to the one-peptide class IId bacteriocin encoded by *salL*. Furthermore, the regulatory and export systems of corresponding salivaricin locus are analogous to those of abp118 and salivaricin P, thereby highlighting the ability of these systems to recognise and produce various antimicrobial peptides.

Discussion

CGH analysis revealed a high level of intraspecies diversity within the intestinal *L. salivarius* isolates employed when compared to the genome of *L. salivarius* UCC118, largely consistent with a recent survey of the genomic diversity of 33 *L. salivarius* strains (28).

462 Bacteriocin production is a megaplasmid encoded feature of importance for
463 the probiotic functionality of *L. salivarius* UCC118 (5, 6) and CGH confirmed the
464 presence of *repA*-type megaplasmids in each of the test strains investigated in this
465 study. The megaplasmids of DPC6488 and DPC6196 were considerably smaller in
466 size than pMP118, notably, a 67 kb non-functional conjugation transfer locus present
467 in UCC118 (10) is absent in these strains. Indeed, this locus is also absent in
468 DPC6502 despite the fact that it harbours a megaplasmid similar in size to pMP118.
469 This indicates a considerable number of additional megaplasmid-encoded features in
470 DPC6502, which also is the case for DPC6027 and DPC6189 which harbour
471 megaplasmids considerably larger than pMP118. CGH analysis of the bacteriocin
472 locus in the porcine salivaricin P-producing test strain DPC6005 produced results
473 which were consistent with subsequent sequence analysis. This revealed that the
474 salivaricin P gene cluster was organised in a similar arrangement to the genetic locus
475 of abp118 (13, 27), with the most notable difference between the genes encoding the
476 induction peptides (60% identity). It was not surprising that the N-terminal region of
477 the corresponding sensory histidine kinase genes also varied slightly (93% identity).
478 Similar variations were previously observed between the regulatory operons of the *pln*
479 loci of *Lactobacillus plantarum* C11 and *L. plantarum* NC8 which are also otherwise
480 highly homologous (19). Indeed, similar levels of variability were observed with
481 respect to the bacteriocin locus of all of the salivaricin P-producing porcine isolates,
482 relative to UCC118, indicating that they may all be derived from a single ancestor.
483 CGH also revealed that the bacteriocin-negative phenotype of the human isolate
484 DPC6196 is likely due to a genetic defect in the bacteriocin transport system in this
485 isolate.

Sequence analysis of the salivaricin locus of strain DPC6488 further validated the CGH data. Although the genes involved in regulation and transport of abp118 were highly conserved in DPC6488, this strain harbours structural genes for the production of two novel antimicrobials, a two-component class IIb bacteriocin salivaricin T, and a one-peptide class IId bacteriocin salivaricin L. It is understandable that strain DPC6488 was previously mistakenly characterised as a salivaricin P producer as the primers designed to amplify the bacteriocin structural genes were complementary to the sequence encoding the double-glycine leader of Sln1 and the immunity genes of salivaricin P, which share considerable identity with the leader sequence of SalTα and the respective immunity genes of the corresponding salivaricin locus. Notably, the porcine isolate DPC6502, which was also previously characterised as a salivaricin-P producer, lacked all abp118-related homologues and so the Bac^{+} phenotype of this strain will be the basis of further investigation.

The leader sequence of the SalTα precursor is almost identical to that of the Abp118α and Sln1 prepeptides (95% identity, with just one conservative aa difference [I/V] in 19), which may be a requirement for the efficient processing and export of the mature active peptide by the conserved ABC transporter. Indeed, while the mature salivaricin T peptides most closely resembled the component peptides of thermophilin 13 (20), the proteins involved in providing immunity to, regulating production of and transporting these peptides are analogous to those of abp118. However, it should be noted that, at present, the thermophilin 13 gene cluster has not been reported. The presence of two putative immunity genes downstream of each of the salivaricin T structural genes is interesting and this fact, combined with the individual activity of each of the component peptides, may indicate that salivaricin T evolved from two synergistically acting one-peptide bacteriocins. Interestingly, the one-peptide class IId

511 bacteriocins cerein 7B and sakacin Q with which salivaricin L shares weak homology
512 are also simultaneously produced with cerein 7A and sakacin P by their respective
513 producing strains *B. cereus* and *L. sakei* (21, 25). As observed with salivaricin T and
514 salivaricin L, neither of these pairs of simultaneously produced bacteriocins exhibit
515 synergistic activity. Moreover, salivaricin L exhibited antagonistic activity to the
516 gastrointestinal pathogen *L. monocytogenes*, with an MIC_{50} of 20 µM.
517 *L. salivarius* DPC6488 was selectively isolated from the neonatal faecal
518 population as a consequence of its antimicrobial phenotype. Therefore, it is tempting
519 to suggest that salivaricin T and salivaricin L production may be an important
520 mechanism for host colonisation and prevalence of the producing strain allowing it to
521 outcompete closely-related populations within the intestine. Notwithstanding this
522 hypothesis, the apparent ability of *L. salivarius* to produce very differing bacteriocins
523 using the same cellular machinery suggests a hitherto unknown versatility with
524 respect to production of this dominant probiotic trait.
525

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Figure legends

Figure 1. Comparative representation of the abp118 and salivaricin T gene clusters. The bacteriocin structural and predicted immunity genes are indicated by the colours black and grey with a black outline, respectively. The homologues of the putative protein products encoded by the salivaricin T gene cluster are outlined in Table 3. The most notable regions of similarity between the respective structural and immunity genes are highlighted by the black dashed lines.

Figure 2. Graphical representation of the genetic diversity of the bacteriocin locus within *L. salivarius* test strains as determined by aCGH. Black, blue and yellow regions represent absence, conservation or overrepresentation of CDS, respectively, corresponding to the colour legend.

Figure 3. Nucleotide sequence and deduced peptide sequence of the salivaticin T structural genes and the immunity genes of salivaricin locus of *L. salivarius* DPC6488. (A) The 1087-bp DNA sequence shown encodes the component peptides of salivaricin T produced by *L. salivarius* 6488 (SalTα and SalTβ) together with their leader sequences (underlined). The GG-processing sites are indicated by bold triangles (▲). Two putative immunity genes are present downstream of *slnTα* and *slnTβ*. Putative ribosome binding sites (RBS), start and stop codons are indicated in bold, start codons are also underlined. An inverted repeat sequence (indicated by arrows) typical of a Rho-independent terminator sequence was identified downstream of *slnTβ* also indicated in bold. (B) Alignment of salivaricin T component precursor peptides with those of abp118, acidocin LF221 A, lactacin F, and thermophilin 13. The leader sequences of the Abp118α and SalTα prepeptides and SalTβ and LafX

689 prepeptides share 95% and 59% identity, respectively. The SalTα propeptide displays
690 greatest identity (46%) with ThmA and their respective complementary peptides,
691 SalTβ and ThmB share 33% identity. The SalTβ propeptide shares greatest identity
692 (41%) with acidocin LF221 A, the partial available sequence of the LF221 A
693 complementary peptide shares 24% identity with the propeptide of SalTα.
694
695 **Figure 4.** MALDI-TOF MS data for purified SalTα and SlaTβ and individual and
696 combined antimicrobial activity of SalTα and SlaTβ against *L. delbrueckii* subsp.
697 *bulgaricus* LMG6901.
698
699 **Figure 5.** Inhibitory effect of synthetic salivaricin L on the growth of indicator strains
700 *Listeria innocua* DPC3572 (A), and *Listeria monocytogenes* NCTC 11994 (B) at
701 concentrations of 0 µM (♦), 1.0 µM (□), 5.0 µM (▲), 10.0 µM (○), 15.0 µM (×), 20.0
702 µM (∆) and 50.0 µM (◊). Error bars represent standard deviations based on triplicate
703 data.

704 **Table 1.** Bacterial strains used in this study

Source	Strain	Relevant features	Reference
	Lactobacillus delbrueckii subsp. bulgaricus LMG 6901	Indicator strain	(2)
	Listeria innocua DPC3572	Indicator strain	(2)
Human	*Lactobacillus salivarius* UCC118	Abp118 producer	(13)
	Lactobacillus salivarius DPC6196	Harbours bacteriocin structural genes	(2)
	Lactobacillus salivarius DPC6488	Produces salivaricin T and salivaricin L	(23)
Porcine	*Lactobacillus salivarius* DPC6502	Bacteriocin producer	(23)
	Lactobacillus salivarius 7.3	Salivaricin P producer	(2)
	Lactobacillus salivarius DPC6189	Salivaricin P producer	(2)
	Lactobacillus salivarius DPC6027	Salivaricin P producer	(2)
	Lactobacillus salivarius DPC6005	Salivaricin P producer	(2)

705

706 **Table 2.** Megaplasmid content of intestinal *L. salivarius* strains

Strain	**UCC118**	**DPC6196**	**DPC6488**	**DPC6502**	**DPC6027**	**DPC6189**	**7.3**	**DPC6005**
Approximate length of circular megaplasmids (kb)	242	180	195	242	320	360	195	242
Additional plasmid content								
Approximate length of linear megaplasmids (kb)	-	-	-	-	-	195	195	-
Approximate length of smaller plasmids (kb)	44, 20	48	30, 20	-	-	-	-	-

707

Table 3. Homologues of the deduced proteins encoded by the salivaricin T bacteriocin locus of *L. salivarius* DPC6488

ORF (gene)	Size (aa)	Function	Homologue	Identity (%)[a]	Reference
ORF 1 (*slnT1*)	65	Conserved hypothetical protein	Conserved hypothetical protein of *L. salivarius* DSM20555	95 [62/65]	EEJ73426[b]
ORF 2 (*slnT2*)	87	Conserved hypothetical protein	Conserved hypothetical protein of *L. salivarius* DSM20555	100 [87/87]	EEJ73427[b]
ORF 3 (*slnT3*)	57	Bacteriocin-like prepeptide	Salivaricin B prepeptide	98 [56/57]	(4)
ORF 4 (*slnT4*)	89	Bacteriocin-like prepeptide	LSL_1918 of *L. salivarius* UCC118	98 [83/85]	(5)
ORF 5 (*slnTα*)	80	Salivaricin T prepeptide SalT alpha	ThmA, amphipathic pore-forming peptide precursor of *S. thermophilus*	45 [35/77]	(20)
ORF 6 (*slnT IM1*)	59	Putative bacteriocin immunity protein	Abp118 IM (LSL_1915) of *L. salivarius* UCC118	82 [45/55]	(13)
ORF 7 (*slnTβ*)	75	Salivaricin T prepeptide SalT beta	Acidocin LF221A produced by *L. gasseri*	46 [31/67]	(18)
ORF 8 (*slnT IM2*)	54	Putative bacteriocin immunity protein	Abp118 IM (LSL_1915) of *L. salivarius* UCC118	76 [38/50]	(13)
ORF 9 (*slnT IP*)	38	Putative induction peptide	Abp118 IP (LSL_1914) of *L. salivarius* UCC118	100 [38/38]	(13)
ORF 10 (*slnT K*)	429	Sensory transduction histidine kinase	AbpK of *L. salivarius* UCC118	99 [424/429]	(13)
ORF 11 (*slnT R*)	265	Response regulator	Salivaricin response regulator of strain CECT5713/ (AbpR 94%)	95 [249/263]	ADJ79880[b]
ORF12	41	Hypothetical protein	no homologues		
ORF 13 (*Sln L*)	59	Bacteriocin-like prepeptide	Putative bacteriocin of Streptococcus sp. C105	60 [35/60]	EFX55741[b]
ORF 14	74	Hypothetical protein	no homologues		
ORF 15 (*slnT T*)	719	Salivaricin T ABC-transporter protein	AbpT (LSL_1910) of *L. salivarius* UCC118	99 [709/719]	(13)
ORF 16 (*slnT D*)	384	Salivaricin T export accessory protein	AbpD (LSL_1909) of *L. salivarius* UCC118	97 [370/384]	(13)
ORF 17	63	Truncated transposase	Transposase ISLasa2b, IS1223 family (LSL_1957)	97 [59/61]	(5)
ORF 18	246	Truncated transposase	Transposase ISLasa2b, IS1223 family (LSL_1957)	94 [231/246]	(5)
ORF 19	172	Transposase	IS1223 family transposase (LSL_1958, LSL_0049) of UCC118	98 [169/172]	(5)
ORF 20	273	Hypothetical protein	Conserved hypothetical protein of *L. salivarius* CECT5713	95 [257/270]	ADJ79877[b]

[a]Percentage identity was determined using BLAST

[b]Accession number of sequence directly submitted to EMBL Database