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RESEARCH ARTICLE

Bactofencin A, a New Type of Cationic Bacteriocin with Unusual Immunity

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ABSTRACT **Bacteriocin production is an important probiotic trait of intestinal bacteria. In this study, we identify a new type of bacteriocin, bactofencin A, produced by a porcine intestinal isolate *Lactobacillus salivarius* DPC6502, and assess its potency against pathogenic species including *Staphylococcus aureus* and *Listeria monocytogenes*. Genome sequencing of the bacteriocin producer revealed *bfnA*, which encodes the mature and highly basic (pI 10.59), 22-amino-acid defensin-like peptide. Matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectral analysis determined that bactofencin A has a molecular mass of 2,782 Da and contains two cysteine residues that form an intramolecular disulfide bond. Although an ABC transporter and transport accessory protein were also present within the bacteriocin gene cluster, a classical bacteriocin immunity gene was not detected. Interestingly, a *dltB* homologue was identified downstream of *bfnA*. DltB is usually encoded within the *dlt* operon of many Gram-positive bacteria. It is responsible for D-alanylation of teichoic acids in the cell wall and has previously been associated with bacterial resistance to cationic antimicrobial peptides. Heterologous expression of this gene conferred bactofencin A-specific immunity on sensitive strains of *L. salivarius* and *S. aureus* (although not *L. monocytogenes*), establishing its role in bacteriocin immunity. An analysis of the distribution of *bfnA* revealed that it was present in four additional isolates derived from porcine origin and absent from five human isolates, suggesting that its distribution is host specific. Given its novelty, we anticipate that bactofencin A represents the prototype of a new class of bacteriocins characterized as being cationic, with a DltB homologue providing a cognate immunity function.**

IMPORTANCE **This study describes the identification, purification, and characterization of bactofencin A, a novel type of bacteriocin produced by *L. salivarius* DPC6502. Interestingly, bactofencin A is not similar to any other known bacteriocin but instead shares similarity with eukaryotic cationic antimicrobial peptides, and here, we demonstrate that it inhibits two medically significant pathogens. Genome sequence analysis of the producing strain also revealed the presence of an atypical *dltB* homologue in the bacteriocin gene cluster, which was lacking a classical bacteriocin immunity gene. Furthermore, cloning this gene rendered sensitive strains resistant to the bacteriocin, thereby establishing its role in providing cognate bacteriocin immunity. Four additional *L. salivarius* isolates, also of porcine origin, were found to contain the bacteriocin biosynthesis genes and successfully produced bactofencin A, while these genes were absent from five human-derived strains investigated.**

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The genus *Lactobacillus* consists of more than 145 species whose habitats range from soil and plants to the mammalian gastrointestinal tract (GIT) (1). These bacteria produce a rich diversity of bacteriocins in terms of structure and mode of action, varying from extensively posttranslationally modified lantibiotics such as plantaricin C (2) to large, unmodified, heat-labile proteins such as helveticin J (3).

Lactobacillus salivarius is a species particularly associated with the mammalian GIT (4–7), and many associated probiotic attributes, including favorable immunomodulatory, antiinflammatory, and antiinfective properties, have been associated with specific strains (8). Indeed, due to its positive immunomodulatory and potent antimicrobial activity, one strain of *L. salivarius* was among six strains chosen to formulate a multispecies probiotic for combating disease in critically ill patients (9). The *L. salivarius* component of another five-strain probiotic combination, *L. salivarius* DPC6005, outcompeted four coadministered strains to dominate within the porcine ileal digesta and mucosa (10). In addition, the antistaphylococcal activity of *L. salivarius* CECT 5713, isolated from a mother and child pair (7), indicated that this strain is an alternative to conventional antibiotics in the treatment of infectious mastitis in women during lactation (11, 12). Further studies with this strain have highlighted its immune-modulatory properties and its safety with respect to human consumption (13–15).

The range of bacteriocins produced by *L. salivarius* extends from the class IIa pediocin-like bacteriocins OR-7 and L-1077 (16, 17) to the two-component class IIb bacteriocins abp118, salivari-

cin P, salivaricin T, and variants (4, 18–23) and the class IId linear non-pediocin-like bacteriocins, such as salivaricin B and salivaricin L (24, 21). Each of these bacteriocins has demonstrated inhibitory activity toward medically significant pathogens. Notably, purified bacteriocin OR-7 and L-1077 have provided *in vivo* protection against *Campylobacter jejuni* and *Salmonella enterica* serovar Enteritidis infection in chickens (16, 17). Moreover, Corr et al. demonstrated abp118-mediated protection against *Listeria monocytogenes* infection in mice upon administration of the producing strain *L. salivarius* UCC118, thereby substantiating the role of bacteriocins in mediating a probiotic effect (61). It is feasible that these bacteriocins could be exploited for the therapeutic manipulation of the intestinal flora. Indeed, the influence of abp118 on the composition of the gut flora of diet-induced obese (DIO) mice and pigs following the administration of the producing strain has also been demonstrated recently (25, 26).

In this study, we describe an unusual bacteriocin, bactofencin A, produced by the porcine intestinal isolate *L. salivarius* DPC6502. The bacteriocin locus encodes a highly basic antimicrobial peptide of just 22 amino acid residues, and in this respect, bactofencin A bears a close resemblance to certain eukaryotic cationic antimicrobial peptides. Moreover, a DltB homologue confers on the producing strain specific immunity to bactofencin A, suggesting a role for teichoic acid in the immunity mechanism. Genes encoding this bacteriocin have been identified in several porcine isolates but appear to be absent from human *L. salivarius* isolates.

RESULTS

The isolation of *L. salivarius* DPC6502, which has broad-spectrum antimicrobial activity, from porcine jejunal digesta has been described previously (27). While PCR assays initially suggested that this strain produces a variant of abp118, recent array-based comparative genomic hybridization (aCGH) analyses revealed the absence of abp118-related homologues in strain DPC6502 (21). Indeed, this strain was the most divergent of seven *L. salivarius* test strains when compared with the genome of the reference strain *L. salivarius* UCC118 (28), exhibiting just 78% conservation of strain UCC118-specific gene content (21). Its potent antimicrobial activity and considerable genetic divergence led to the further characterization of strain DPC6502 and the associated antimicrobial activity.

Characterization of the antimicrobial phenotype of *L. salivarius* DPC6502. The peptide responsible for the antimicrobial activity of *L. salivarius* DPC6502 was purified from an overnight culture of the strain using cation exchange followed by reversed-phase chromatography. Subsequent matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) analysis revealed an associated mass of 2,782 Da in the active fractions (Fig. 1). Edman analysis revealed the N-terminal sequence KRKXHRXRVYNNGMPTGMYRYM, where "X" at positions 4 and 7 indicates blank cycles for which no amino acid derivative was detected. A homology search did not identify any similar sequences in protein databases. The purified bacteriocin peptide inhibited closely related lactobacilli, as well as *Staphylococcus aureus* and, to a lesser extent, *Listeria innocua* (Fig. 1).

Considering the unusual nature of this bacteriocin, the genome of the producing strain *L. salivarius* DPC6502 was sequenced to identify the corresponding gene cluster.

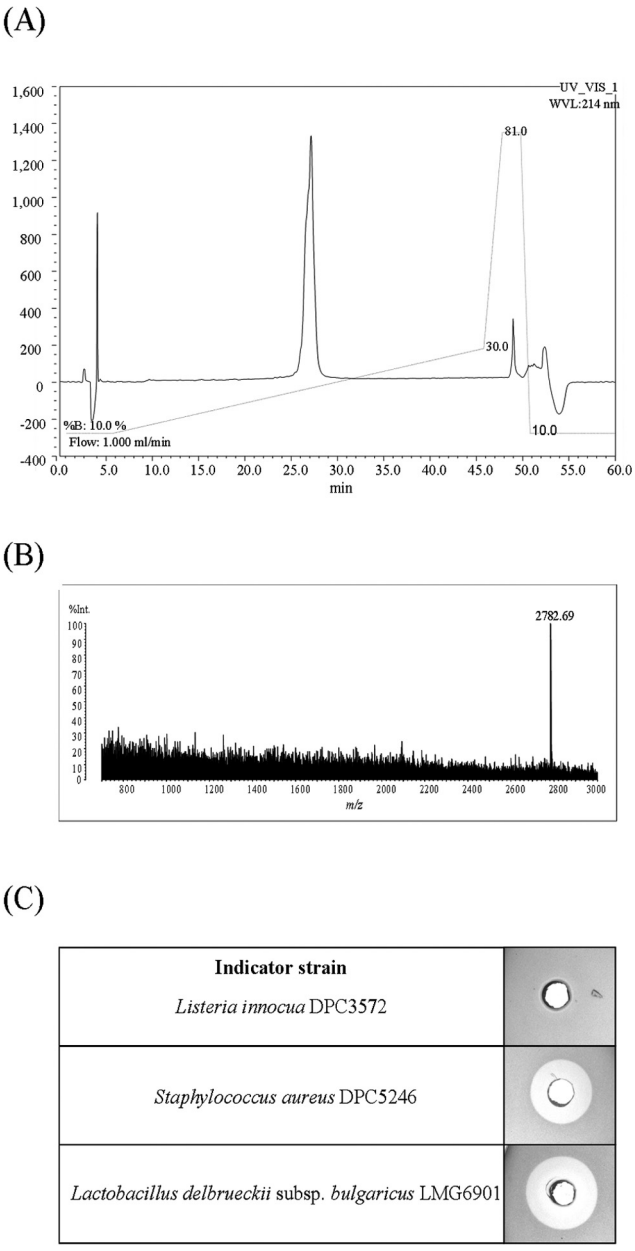

FIG 1 HPLC profile (A), MALDI-TOF MS data (B), and antimicrobial activity (C) of purified bactofencin A. WVL, wavelength.

Characterization of the bacteriocin locus in the genome of *L. salivarius* DPC6502. Scanning of the entire draft genome sequence for a gene corresponding to the N-terminally derived amino acid sequence revealed a chromosomally located open reading frame (ORF), DLSL_0050, present on a gene cluster which was absent from the publically available *L. salivarius* genome sequences. The unidentified amino acids at positions 4 and 7 in the sequence determined by Edman degradation were, on the basis of the corresponding codons, found to be lysine and cysteine residues, respectively (Fig. 2). The bacteriocin structural gene consists of 159 nucleotides and is predicted to encode a 53-amino-acid (aa) precursor peptide comprised of a 31-aa double-glycine leader sequence and a 22-aa propeptide sequence which differed from that

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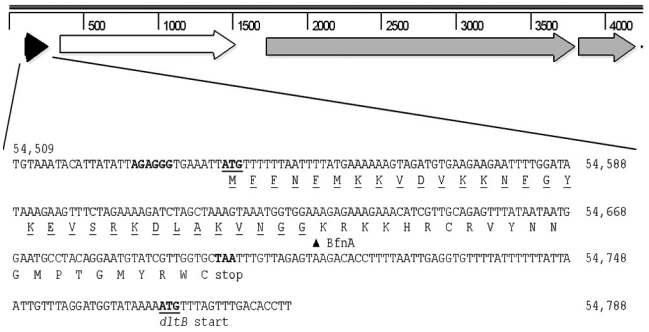

FIG 2 Nucleotide sequence and deduced peptide sequence of the structural gene of bactofencin A. The leader sequence is underlined, and the GG-processing site is indicated by an arrowhead. The bacteriocin structural gene and predicted immunity gene and transport genes are indicated by black, white, and gray arrows, respectively.

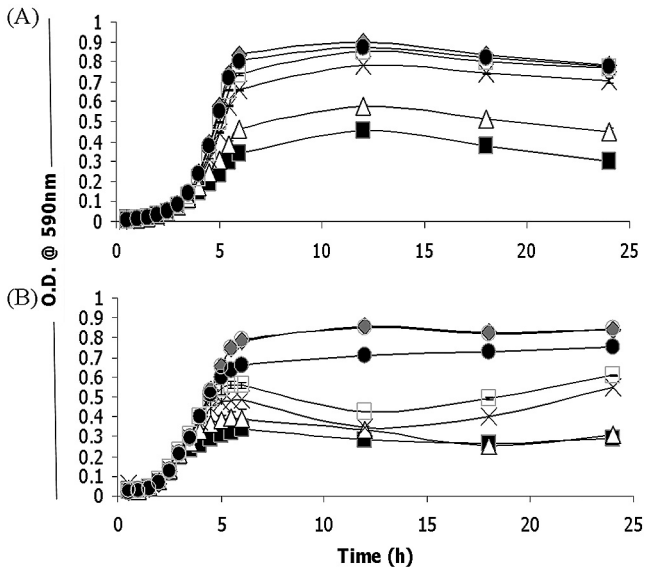

FIG 3 Inhibitory effect of synthetic bactofencin A on the growth of the indicator strains *L. monocytogenes* NCTC 11994 (A) and *S. aureus* DPC5246 (B) at concentrations of 0 μM (gray diamonds), 0.05 μM (open circles), 0.1 μM (black circles), 0.5 μM (open squares), 1.0 μM (×), 5.0 μM (open trangles), and 10.0 μM (black squares). Error bars represent standard deviations based on triplicate data.

derived by peptide sequencing with respect to the two most C-terminally located residues (Fig. 2). The predicted mass of the mature translation product (2,785 Da) differed from that determined by MALDI-TOF MS analysis of the active peptide (2,782 Da) by 3 Da. MS analysis revealed an increase of 2 Da (2,784 Da) in the mass of the active peptide when the cysteine residues were in a reduced state (after treatment with dithreitol [DTT] and iodoacetamide), indicating that an intramolecular disulfide bond is formed between Cys7 and Cys22. A search of the BLAST database did not identify any homologues for this ORF, indicating that this peptide is unlike any previously characterized bacteriocins. However, a search of the antimicrobial peptide database (29) revealed that bactofencin A shares the greatest similarity (42%) with a plant antimicrobial peptide (AMP) isolated from extracts of the seed of *Impatiens balsamina* (Ib-AMP3) which displays both antibacterial and antifungal properties (30). In consequence of the greater similarity of this bacteriocin to cationic peptides of eukaryotic origin, it was designated bactofencin A. Despite its similarity to Ib-AMP3, neither *L. salivarius* DPC6502 nor the purified bactofencin A peptide exhibited antifungal activity against the indicator strains investigated (data not shown).

The ORF immediately downstream from the bacteriocin structural gene (*bfnA*) encodes a putative product of 396 aa which shares 74% identity with an operon-encoded D-alanyl transfer protein (DltB) of *Pediococcus pentosaceus* ATCC 25745 (accession no. YP_805052) and 65% identity with the operon-encoded DltB of *L. salivarius* UCC118 (LSL_0891). DltB is a transmembrane protein responsible for the transfer of activated D-alanine across the cytoplasmic membrane, which is indispensable for the D-alanyl esterification of teichoic acids (31, 32). Interestingly, D-alanylation of teichoic acids has previously been found to contribute to bacterial resistance to cationic antimicrobial peptides due to the resultant reduction in the net negative charge generated in the bacterial cell wall, thereby attenuating essential electrostatic interactions (32–36). A second DltB-encoding gene (DLSL_0976), present in a *dlt* operon (DLSL_0974 to DLSL_0978), is also located on the chromosome of *L. salivarius* DPC6502, the product of which shares 99% identity with DltB of UCC118 (encoded by LSL_0891) and 65% identity with the deduced protein product of DLSL_0051. The ORFs downstream from the *dltB* homologue encode a putative bacteriocin ABC transporter (DLSL_0052) and a bacteriocin transport accessory protein (DLSL_0053). The deduced protein sequence of the ABC transporter contains an N-terminal peptidase C39 domain of 139 aa which contains a conserved cysteine motif and histidine motif characteristic of the putative catalytic site responsible for the cleavage of double-glycine leader sequences (37, 38). Thus, these genes encode proteins which are probably responsible for the processing and secretion of mature active bactofencin A. This putative bacteriocin transport system likely completes the bacteriocin gene cluster (approximately 4 kb), as the adjacent ORFs, which are conserved in UCC118 (LSL_0035 to LSL_0042), display similarity to the WalRK (YycGF) regulon responsible for the regulation of bacterial cell wall metabolism of low-G+C Gram-positive bacteria (39).

Bactofencin A is active at micromolar concentrations. The identification of the structural gene and deduced peptide sequence facilitated the production of synthetic bactofencin A. MS analysis revealed a mass of 2,784 Da for the synthetic form of the peptide, which displayed anti-*Listeria* and anti-*S. aureus* activity that was similar to that of the purified natural bactofencin A peptide (data not shown). This suggests that the disulfide bond of the natural form of bactofencin A is not crucial for activity. In consequence of this and of the fact that the synthetic approach provided access to larger quantities of peptide, the synthetic bactofencin A peptide was employed for further antimicrobial activity assays, which on this occasion took the form of more-sensitive broth-based MIC_{50} assays. These investigations revealed that, although concentrations of up to 50 μM of synthetic bactofencin A did not inhibit *Escherichia coli*, *Cronobacter sakazakii*, *Salmonella*, or any of the fungal indicator strains employed, concentrations of 1 to 5 μM were sufficient to inhibit the growth of both *S. aureus* DPC5246 and *L. monocytogenes* NCTC 11994 by 50% (Fig. 3). Furthermore, the bactericidal effect of bactofencin A was demon-

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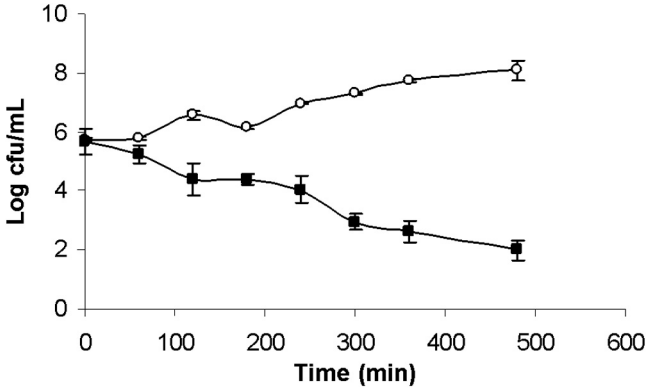

FIG 4 Viability of *S. aureus* DPC5246 treated with (black squares) and without (open circles) synthetic bactofencin A as a function of time. These results represent the average of three independent experiments. Error bars represent standard errors based on triplicate data.

strated by a 4-log reduction in viable *S. aureus* DPC5246 cells following an 8-h incubation with the bacteriocin (Fig. 4).

A DltB homologue is responsible for bactofencin A-specific immunity. Typically, the unmodified class II bacteriocin structural genes are cotranscribed with an ORF encoding a cognate immunity protein, usually 50 to 150 aa in size and located downstream of the structural gene, which provides self-protection for the producing strain. To determine whether the DltB homologue encoded by the ORF immediately downstream of *bfnA*, designated *bfnI*, has a role in bactofencin A immunity, an expression plasmid (pEOS01) harboring *bfnI* was constructed. When introduced into the sensitive indicator strains *L. monocytogenes* NCTC11994 and *S. aureus* DPC5246, a statistically significant increase in resistance to bactofencin A, relative to the resistance of the corresponding isogenic control strains harboring the pNZ44 plasmid, was evident for transformants of the *S. aureus* DPC5246 indicator strain (Fig. 5) but not for those of the *L. monocytogenes* NCTC11994 strain (data not shown). Moreover, introducing the pEOS01 construct into the more-similar, yet sensitive strain *L. salivarius* UCC118 resulted in almost-complete immunity to the bacteriocin for the corresponding transformants relative to the control (Fig. 5B). However, this resistance was specific for bactofencin A, as these clones did not exhibit enhanced resistance when exposed to a range of cationic antimicrobial peptides, suggesting that the protection provided is not due to a general impact on cell wall charge (Fig. 6).

The results of cytochrome *c* binding assays also correlated with these results. Cytochrome *c* is a highly positively charged protein (pI 10), readily detected at 530 nm, whose binding is dependent on the net negative cell surface charge. In a control study, following a 10-min exposure, the level of unbound cytochrome *c* detected in the cell-free supernatant (CFS) of the *dltA*-deficient *S. aureus* Sa113 mutant was considerably lower than the level in the CFS of the wild-type strain. This indicates an increase in the net negative cell surface charge of the mutant relative to that of the wild-type

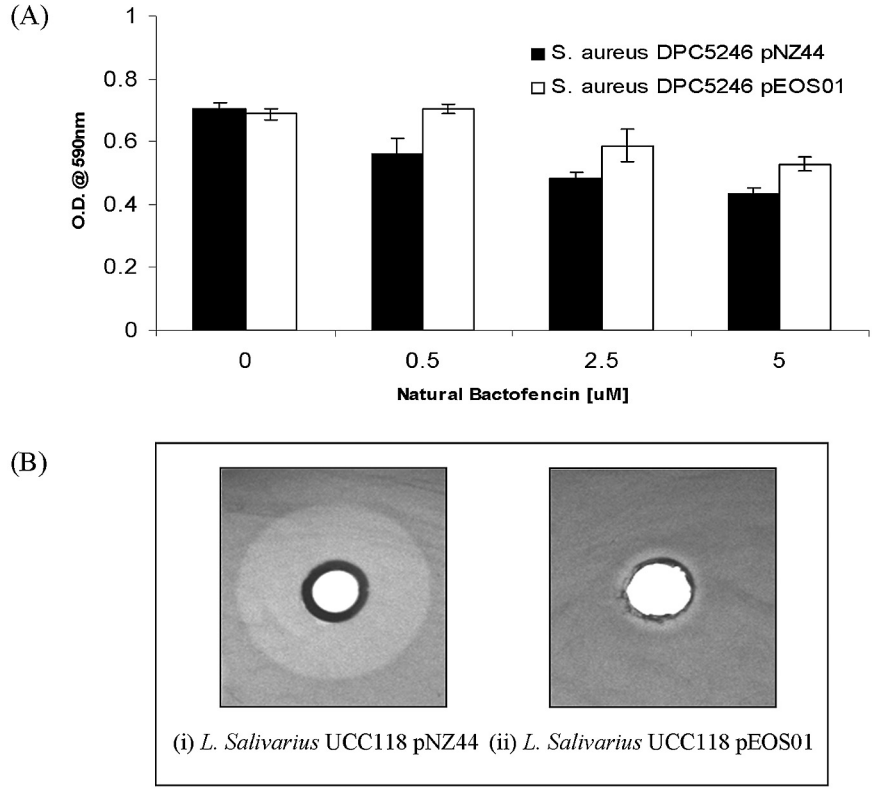

FIG 5 (A) *S. aureus* DPC5246 containing pEOS01 exhibits significantly enhanced resistance to bactofencin A relative to the resistance of the isogenic control strain at concentrations of 0.5 μM ($P < 0.01$), 2.5 μM ($P < 0.05$), and 5 μM ($P < 0.05$) following 24 h of incubation with the peptide. Error bars represent standard deviations based on triplicate data. (B) *L. salivarius* UCC118 containing pEOS01 (ii) also exhibits enhanced resistance to bactofencin A relative to the resistance of the respective isogenic control strain harboring pNZ44 (i).

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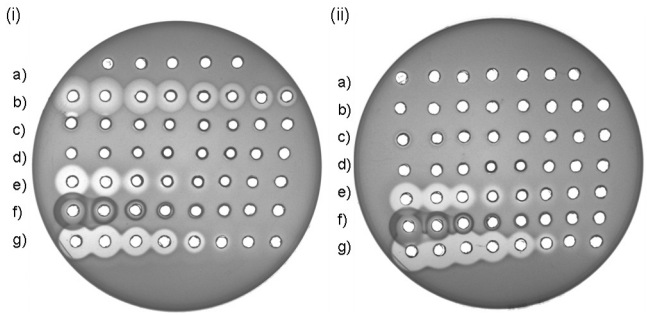

FIG 6 The specific immunity of *L. salivarius* UCC118 containing pEOS01 to bactofencin A (ii) (b) relative to the resistance of *L. salivarius* UCC118 containing pNZ44 (i) (b) was not extended to the additional cationic antimicrobial peptides investigated, gramicidin (a), protegrin-1 (c), magainin II (d), (e) polymyxin B (e), ε-polylysine (f), and nisin (g).

strain, due to the associated decrease in D-alanylation of teichoic acids (Fig. 7). However, the affinity for cytochrome *c* of *S. aureus* DPC5246 or *L. salivarius* UCC118 containing pEOS01 did not differ from that of the respective control (Fig. 7).

The bactofencin A locus is a novel hypervariable gene cluster characteristic of *L. salivarius* of porcine origin. The production of similar or identical bacteriocins is frequent among genetically distinct strains or even species of lactic acid bacteria (LAB) (6, 40–42). Indeed, the genetic determinants for abp118-like bacteriocins are among the relatively high content of hypervariable gene clusters of *L. salivarius* (6, 21, 43). To investigate whether the bactofencin A locus described in this study is similarly among the hypervariable loci of the flexible gene pool of *L. salivarius*, we investigated eight additional *L. salivarius* isolates, four of human and four of porcine intestinal origin (Table 1), for the presence of the bactofencin A structural gene. A PCR product corresponding to this gene could not be generated from the genomic DNA of the four strains of human origin, namely, DPC6488, DPC6196, DPC6107, and DPC6095. Cross-immunity assays also revealed that each of these isolates was sensitive to the bactofencin A-producing strain. However, all four additional porcine strains (*L. salivarius* DPC6005, DPC6027, DPC6189, and 7.3) were immune to bactofencin A and PCR positive for *bfnA*. Sequencing of the PCR products generated confirmed 100% identity with the bactofencin A structural gene of DPC6502 in each case. Subsequent purification and MS analysis confirmed bactofencin A production in these strains.

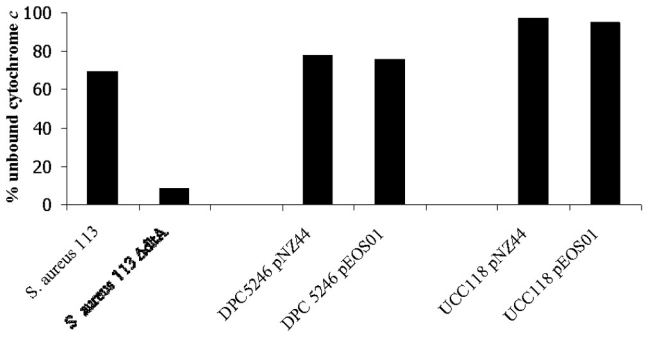

FIG 7 Comparison of relative net cell surface charge. The *dltA*-deficient *S. aureus* Sa113 experimental control has a higher affinity for cytochrome *c* than wild-type *S. aureus* Sa113 due to a decrease in D-alanylation of teichoic acids and a resultant increase in the net negative cell surface charge. However, the expression of BfnI does not influence the affinity of *S. aureus* DPC5246 or *L. salivarius* UCC118 for cytochrome *c* relative to the affinities of their respective controls.

DISCUSSION

Sequence analysis of the *L. salivarius* DPC6502 genome revealed that the bactofencin A structural gene is encoded on a gene cluster of approximately 4 kb which is not present in the well-characterized *L. salivarius* UCC118 strain. While the leader sequence **–VS**RK**DL**AK**V**N**GG** of the bacteriocin prepeptide is unusually long (31 aa), it conforms with the consensus for double-glycine leaders (as highlighted in boldface) (38). The fact that its bioactivity did not require extensive posttranslational modification of the peptide and the absence of the characteristic YGNGV consensus motif of pediocin-like class IIa bacteriocins suggest that this peptide belongs to the diverse class IId bacteriocins of Gram-positive bacteria (44). Class IId consists of a heterogenous collection of bacteriocins which cannot be assigned to any of the other known bacteriocin subgroups. The mature bactofencin A peptide is highly basic, containing eight positively charged residues that are largely concentrated at the N terminus, which may be involved in mediating the initial binding of the bacteriocin to target cells via electrostatic interaction. Database searches have revealed that this peptide does not share significant homology with previously characterized bacteriocins but more closely resembles eukaryotic antimicrobial peptides. It would thus appear that bactofencin A is the first of a novel group of bacteriocins.

Although a previous assessment of the antimicrobial activity of the producing strain, using agar well diffusion assays with neutralized CFS, revealed a broad spectrum of inhibition which included 22 of 62 indicator strains (27), this activity was found to be predominantly against closely related strains of LAB. The availability of purified bactofencin A in this study established the *in vitro* efficacy of the bacteriocin, revealing MIC_{50} values of 1 to 5 μM against the pathogenic indicator strains *L. monocytogenes* and *S. aureus*, as well as the bactericidal nature of this antimicrobial activity. This activity is comparable to that of the two-component lantibiotic lacticin 3147, which displayed a MIC_{50} of 7 to 8 μM for the bovine mastitis isolate *S. aureus* DPC5245 (45). However, the fact that bactofencin A is an unmodified bacteriocin and, thus, can be readily generated in large quantities in a synthetic form may make this novel bacteriocin a more-favorable alternative to antibiotics for animal husbandry-related applications.

Despite its uncharacteristically large size (396 aa) relative to the sizes of cognate bacteriocin immunity proteins (30 to 100 aa), the expression of *bfnI* enhanced the resistance of two sensitive indicator species to bactofencin A, conferring almost-complete immunity on *L. salivarius* UCC118. However, a statistically significant increase in bactofencin A resistance was not observed for the *L. monocytogenes* indicator strain, possibly due to the lower sensitivity of this strain to the bacteriocin (Fig. 3A). BfnI shares considerable homology with D-alanyl transfer proteins, which are generally encoded on the *dlt* operon, responsible for the D-alanylation of teichoic acids on the bacterial cell wall. Being predominantly negatively charged, teichoic acids are consequently a major determinant of the cell wall electrostatic interactions. Increased D-alanylation of teichoic acids attenuates such interactions, thereby conferring resistance to cationic antimicrobial peptides (32–36). Interestingly, *S. aureus* organisms harbor-

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TABLE 1 Bacterial strains used in this study

Strain	Relevant features	Reference
Lactobacillus salivarius	UCC118, Abp118 producer, human intestinal isolate	19
Lactobacillus salivarius	DPC6502, bactofencin A producer, porcine intestinal isolate	27
Lactobacillus salivarius	DPC6005, salivaricin P producer, bactofencin A producer, porcine intestinal isolate	4
Lactobacillus salivarius 7.3	Salivaricin P producer, bactofencin A producer, porcine intestinal isolate	4
Lactobacillus salivarius DPC6189	Salivaricin P producer, bactofencin A producer, porcine intestinal isolate	4
Lactobacillus salivarius DPC6027	Salivaricin P producer, bactofencin A producer, porcine intestinal isolate	4
Lactobacillus salivarius DPC6488	Salivaricin T producer, human intestinal isolate	27
Lactobacillus salivarius DPC6196	Bac$^-$, despite harboring bacteriocin structural genes, human intestinal isolate	4
Lactobacillus salivarius DPC6095	Bac$^-$, human intestinal isolate	
Lactobacillus salivarius DPC6107	Bac$^-$, human intestinal isolate	
Escherichia coli XL-1 blue	Intermediate host for DNA manipulations	Stratagene
Microbial indicator strains		
Escherichia coli DH5α		
Escherichia coli	O157:H7 strain P1432, nontoxigenic	62
Cronobacter (Enterobacter) *sakazakii*	NCTC08155	63
Listeria innocua DPC3572	Nonpathogenic	27
Listeria monocytogenes NCTC11994		46
Salmonella enterica serovar Typhimurium DT104		27
Staphylococcus aureus DPC5246	Mastitis isolate	45
Staphylococcus aureus DPC5646	Methicillin resistant	64

ing several copies of the *dlt* operon displayed decreased sensitivity to various cationic antimicrobial peptides (32). However, the resistance mediated by BfnI did not extend to a range of cationic antimicrobials investigated but was exclusive for bactofencin A. Therefore, despite the homology of BfnI to DltB proteins, these results suggest that BfnI does not function to increase the content of esterified D-alanyl of teichoic acids on the bacterial cell surface. To our knowledge, this is the first report of a DltB homologue for which a specific mechanism of bacteriocin immunity has been reported.

The ability of genetically distinct strains and, in several cases, distinct species to produce similar or identical bacteriocins is a common phenomenon of LAB (6, 40–42). It is evident from the results of this study that bactofencin A is among the hypervariable gene clusters of *L. salivarius* and is perhaps exclusively associated with those of porcine intestinal origin. The additional porcine isolates investigated for bactofencin A production in this study also produce a two-component class IIb bacteriocin, salivaricin P (4). Bactofencin A production was previously undetected in antimicrobial analyses of these strains, likely as a consequence of its hydrophilic nature and the potent antimicrobial activity of the coproduced salivaricin P (MIC_{50} of 50 nM for *L. innocua* DPC5372) (4, 46). Notably, previous analyses revealed that both of the bacteriocin-positive human isolates *L. salivarius* UCC118 and DPC6488 were sensitive to the antimicrobial activity of strain DPC6005 (21), despite the considerable homology of the respective putative immunity proteins of abp118, salivaricin T, and salivaricin P produced by these strains. Thus, this was likely a consequence of bactofencin A-mediated activity of strain DPC6005. Furthermore, *L. salivarius* DPC6005 has also demonstrated a competitive advantage within the porcine ileum (10) and was associated with reduced *Salmonella* shedding in pigs when administered as part of a probiotic formulation (47). A combination of bactofencin A and salivaricin P production likely contributed to the superior probiotic properties demonstrated by this strain within the porcine intestine.

In conclusion, a novel bacteriocin with potent anti-*Listeria* and anti-*S. aureus* activity has been identified in *L. salivarius*. Genomic sequence analysis of the producing strain revealed the genetic determinants responsible for bacteriocin production. The amino acid sequence of the cationic bacteriocin did not display significant homology with previously characterized bacteriocins but instead was found to be more similar to eukaryotic antimicrobial peptides. Significantly, this small antimicrobial displayed broad-spectrum antimicrobial activity and antistaphylococcal activity comparable to that of the well-characterized lantibiotic lacticin 3147. Moreover, the wide distribution of bactofencin A among *L. salivarius* isolates of porcine origin may be indicative of a host species-specific trait. We also report the involvement of a DltB homologue in a specific mechanism of bacteriocin immunity.

MATERIALS AND METHODS

Bacterial and fungal strains and culture conditions. The *L. salivarius* strains used in this study are listed in Table 1. All lactobacilli were routinely cultured under anaerobic conditions at 37°C in MRS medium (Difco Laboratories, Detroit, MI), unless otherwise stated. Anaerobic conditions were maintained with the use of anaerobic jars and Anaerocult A gas packs (Merck, Darmstadt, Germany). The *Escherichia coli*, *Cronobacter* (Enterobacter), *Listeria*, and staphylococcal indicator strains employed for antimicrobial characterization (Table 1) were grown aerobically at 37°C in brain heart infusion (BHI) medium (Merck). *E. coli* XL-1 Blue, used as an intermediate host for DNA manipulations, was also grown aerobically at 37°C in LB medium (Merck). Chloramphenicol (Sigma, Poole, United Kingdom) was used in selective medium, being added at concentrations of 20 μg ml^{-1} (*Escherichia coli*) and 5 μg ml^{-1} (*Staphylococcus aureus*, *L. monocytogenes*, and *L. salivarius* strains). The indicator strains employed for antifungal characterization included *Saccharomyces cerevisiae*, *Aspergillus niger*, *Botrytis cinerea*, *Geotrichium candidum*, *Penicillium notatum,* and *Rhizopus stolonifer*. These were maintained aerobically on potato dextrose medium (Merck) at 25°C.

Purification of the hydrophilic antimicrobial peptide produced by *L. salivarius* DPC6502. The antimicrobial peptide was purified from a 2-liter overnight culture of *L. salivarius* DPC6502 grown in MRS medium. The cells were removed by centrifugation at 8,000 × *g* for 15 min, and the

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supernatant applied to a column containing 90 ml SP Sepharose fast-flow cation-exchange resin (GE Healthcare, United Kingdom) previously equilibrated with 20 mM potassium phosphate buffer, pH 2.5, containing 25% acetonitrile. The column was washed with 20 mM potassium phosphate buffer, pH 2.5, containing 25% acetonitrile, 600 mM KCl, and the bioactive peptide was subsequently eluted from the column using 20 mM potassium phosphate buffer, pH 2.5, containing 25% acetonitrile, 1 M KCl. Acetonitrile was removed by rotary evaporation before the sample was applied to a 5-g Strata-E C_{18} SPE column (Phenomenex, Cheshire, United Kingdom) preequilibrated with methanol and water. The column was washed with distilled water, and the peptide eluted with 70% (vol/vol) propan-2-ol containing 0.1% (vol/vol) trifluoroacetic acid (TFA). The propan-2-ol was removed by rotary evaporation, and 2- by 4-ml aliquots of the resultant preparation were applied to a Jupiter proteo reversed-phase high-performance liquid chromatography (RP-HPLC) column (250.0 by 10.0 mm, 4-μm particle size, 90-Å pore size; Phenomenex). The column was preequilibrated with 10% (vol/vol) acetonitrile containing 0.1% (vol/vol) TFA, followed by separation and elution of the bioactive peptide by gradient RP-HPLC using 0.1% (vol/vol) TFA and acetonitrile concentrations that ranged from 10% to 30% (vol/vol), over a period of 5 to 45 min at a flow rate of 2.5 ml min^{-1}. Absorbance was monitored at a wavelength of 214 nm.

Bacteriocin activity was monitored throughout the purification procedure by well diffusion assay using the sensitive indicator strain *Lactobacillus delbrueckii* subsp. *bulgaricus* LMG6901. MALDI-TOF MS (Axima-TOF^2; Shimadzu Biotech, Manchester, United Kingdom) analysis was performed on bioactive fractions as described previously (48). Fractions of interest were further purified by reapplying them to the RP-HPLC column under the conditions described above. N-terminal sequence analysis of the purified antimicrobial peptide by Edman degradation was performed by Aberdeen Proteomics (University of Aberdeen, Scotland). Reduction and alkylation of the cysteine residues were performed using DTT and iodoacetamide (Sigma).

Genome sequencing and analysis. The sequence of the genomic DNA extracted from *L. salivarius* DPC6502 was determined through random shotgun pyrosequencing (Beckman Coulter Genomics, United States). The draft genome assembly was processed using the annotation software package GAMOLA (49). The gene model was determined with Gene Locator and Interpolated Markov ModelER (GLIMMER) 3.02 (50). Sequence similarity analyses were performed using the gapped BLASTp algorithm and the nonredundant database provided by NCBI (ftp://ftp.ncbi.nih.gov/blast/db) (51). The Artemis Comparison Tool (ACT) (52) facilitated comparative analysis of the draft genome assembly of *L. salivarius* DPC6502 with the complete genome sequence of *L. salivarius* UCC118 (28). The genome annotation was manually verified also using the ARTEMIS package. Global whole-genome and megaplasmid alignments were performed using stretcher, available at the EMBOSS server (http://emboss.sourceforge.net/) (53).

Generation of a synthetic analogue of bactofencin A. The bactofencin A peptide was synthesized according to the deduced amino acid sequence of the chromosomally located bacteriocin structural gene (DLSL_0050) using microwave-assisted solid-phase peptide synthesis (MW-SPPS) performed on a CEM Liberty microwave peptide synthesizer using an H-Cys-HMPB-ChemMatrix resin (PCAS Biomatrix, Inc., Quebec, Canada). The synthetic peptide was purified by RP-HPLC as described above. Fractions containing a peptide with the desired molecular mass, as identified by MALDI-TOF MS, were pooled and lyophilized using a Genevac HT 4× evaporator (Genevac Ltd., Ipswitch, United Kingdom). The peptide was dissolved in 70% (vol/vol) propan-2-ol at a concentration of 5 mg ml^{-1} and stored at −20°C. Appropriate dilutions of the peptide in 50 mM potassium phosphate buffer, pH 6.8, were used to determine the specific activity of the synthetic analogue.

Specific activity determination. A microtiter plate assay system was used to determine the MIC_{50} values of the synthetic bactofencin A analogue for a range of pathogenic indicator strains (Table 1). The microtiter plate was first treated with bovine serum albumin (BSA) to prevent adherence of the peptide to the sides of the wells, as described previously (54). Each plate included triplicate assays at each concentration of synthetic bactofencin A examined. Each well contained a total volume of 200 μl, comprised of purified bactofencin A, the first component added to the well, and 150 μl of a 1-in-10 dilution of the indicator culture (A_{590} of 0.1) in BHI broth. Control wells contained medium only (blanks) and untreated indicator culture. The microtiter plate cultures were incubated at 37°C for 24 h, and the optical densities at 590 nm (OD_{590}) recorded at 30-min intervals (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} reading ± 0.05 of the untreated control culture.

To determine whether the antimicrobial activity of bactofencin A is bactericidal or bacteriostatic in nature, the effect of bactofencin A on *S. aureus* DPC5246 was further investigated. Three independent cultures of *S. aureus* DPC5246 were grown overnight at 37°C. 3 replicate 1-ml volumes of sterile double-strength BHI broth medium were inoculated with the test organism to give initial cell numbers of approximately 10^6 CFU/ml. Bactofencin A was added, and the volume made up to 2 ml with sterile distilled water. The bacteriocin was omitted from the control and its volume replaced with sterile water. Samples were removed at intervals, serially diluted, plated on BHI agar, and then incubated aerobically at 37°C.

DNA manipulations, transformation, and plasmid construction. The DltB homologue encoded by DLSL_0051, designated *bfnI*, was amplified by routine PCR using velocity DNA polymerase (Bioline, United Kingdom) and the primer pair *bfnI*F, 5′ CGGGGTACCCCAGGAATGT ATCGTTGGTGC 3′, and *bfnI*R, 5′ CGAGCTCGTTACATACAATTGCA ACCATAC 3′, containing the restriction sites for the KpnI and SacI enzymes (underlined). Digestion facilitated insertion of the resultant product downstream from the constitutive p44 lactococcal promoter in the expression vector pNZ44 (55) to generate pEOS01. The integrity of the insert was confirmed by DNA sequencing using the primer pair pNZ44_for, 5′ TTACAGGTACATCATTCTGTTTGT 3′, and pNZ44_rev, 5′ TGTTTTAACGATTATGCCGATAAC 3′, specific for the pNZ44 multiple cloning site (56). Restriction enzymes and T4 DNA ligase (New England Biolabs, Beverly, MA) were used according to the manufacturer's instructions. PCR products were purified using the Isolate PCR and gel kit (Bioline), and the Qiagen plasmid minikit (Qiagen, West Sussex, United Kingdom) was used to isolate plasmid DNA from *E. coli* transformants. *E. coli*, *S. aureus*, *L. monocytogenes*, and *L. salivarius* were transformed by electroporation in accordance with methods described previously (57–60). The sensitivities of the clones were compared to those of the control strains using the microtiter well-based assay system described above and also by well diffusion assay using 2-fold serial dilutions of bactofencin A prepared in 50 mM potassium phosphate buffer, pH 6.8. Similarly, the clones and their respective controls were assayed against the cationic antimicrobial peptides magainin II, polymyxin B, protegrin-1, ε-polylysine, and nisin, as well as gramicidin D, an antimicrobial of neutral charge.

Comparison of relative net cell surface charges. The cytochrome *c* binding affinity of the *bfnI* clones and their respective isogenic controls were measured as described previously (32). Briefly, mid-log-phase cells (with an OD_{600} of 0.5) were harvested and washed twice with 20 mM MOPS (morpholinepropanesulfonic acid), pH 7. The cells were then resuspended in 0.5 ml 20 mM MOPS, pH 7, and cytochrome *c* from equine heart (Sigma) was added at a final concentration of 0.5 mg ml^{-1}. Following a 10-min incubation at room temperature, the cells were pelleted and the unbound cytochrome *c* present in the supernatant was quantified spectrophotometrically at 530 nm. A control consisting of 0.5 mg ml^{-1} cytochrome *c* in 20 mM MOPS, pH 7 (buffer without cells), was also included as a reference. The absorbance measurements obtained compared with that of the reference were calculated as the absorption ratios (reflecting the bacterial surface charge). A *dltA*-deficient *S. aureus* Sa113

mutant and the corresponding parent strain (32) served as experimental controls. The data are representative of at least three independent studies.

Detection of *bfnA*. The presence of *bfnA* was determined by PCR using template DNA from eight additional genetically distinct intestinal *L. salivarius* isolates (Table 1) and the primer pair *bfnA*F, 5′ CAGTCGAC AATGATCATGATGGAGTAGCG 3′, and *bfnA*R, 5′ GGAAGTAAGTAG GGTTTTGATAAGGTGTC 3′, to amplify a product of 430 nucleotides. This was performed using the Expand high-fidelity PCR system (Roche) according to the manufacturer's instructions. The products derived from PCR were purified and sequenced (Beckman Coulter Genomics), and analysis of DNA sequence data was performed using Lasergene 8 software (DNASTAR, Inc., Madison, WI). Template DNA of *L. salivarius* DPC6502 and *L. salivarius* UCC118 were used as positive and negative controls, respectively. Subsequent purification and MS analyses, as described above, further confirmed positive results.

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