Salivaricin 9, a new lantibiotic produced by *Streptococcus salivarius*

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Salivaricin 9 (Sal9) is a 2560 Da lantibiotic having just 46% amino acid identity with its closest known homologue, the *Streptococcus pyogenes* lantibiotic SA-FF22. The Sal9 locus (designated *siv*) in *Streptococcus salivarius* strain 9 was partially sequenced and localized to an approximately 170 kb megaplasmid, which also harbours the locus for the lantibiotic salivaricin A4. The entire locus was fully characterized in the draft genome sequence of *S. salivarius* strain JIM8780 and shown to consist of eight genes, having the following putative functions: *sivK*, sensor kinase; *sivR*, response regulator; *sivA*, Sal9 precursor peptide; *sivM*, lantibiotic modification enzyme; *sivT*, ABC transporter involved in the export of Sal9 and concomitant cleavage of its leader peptide; and *sivFEG*, encoding lantibiotic self-immunity. Intriguingly, in contrast to strain 9, the *siv* locus was chromosomally located in strain JIM8780 – the first lantibiotic locus shown not to be exclusively plasmid-associated in *S. salivarius*. Sal9-containing extracts specifically induced lantibiotic production in both strain 9 and strain JIM8780, indicating that Sal9 functions as a signal peptide for upregulation of its own biosynthesis. Screening representative strains of three streptococcal species (*S. salivarius*, *S. pyogenes* and *S. mitis*) for *sivA* indicated that it was present only in *S. salivarius*, with 12 of 28 tested *S. salivarius* positive. Since Sal9 was inhibitory to all tested *S. pyogenes* strains it appears to have potential as an important component of the bacteriocin armoury of *S. salivarius* probiotics intended to control *S. pyogenes* infections of the human oral cavity.

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INTRODUCTION

Lantibiotics are ribosomally synthesized antimicrobial peptides that contain characteristic post-translational modifications, typified by the thioether amino acids lanthionine and methyllanthionine, from which the name lantibiotic is derived (Chatterjee *et al.*, 2005; Jung, 1991; Schnell *et al.*, 1988). Approximately 50 lantibiotics have been shown to be produced by Gram-positive bacteria (Patton & van der Donk, 2005), and nisin, the prototype lantibiotic, has been used as a food preservative for more than 40 years (Chatterjee *et al.*, 2005). Other lantibiotics are also beginning to find commercial outlets, such as the proposed use of lacticin 3147 for the prevention and treatment of mastitis in dairy cattle (Twomey *et al.*, 2000).

Abbreviations: BLIS, bacteriocin-like inhibitory substance(s); P-type, producer-type.

The GenBank/EMBL/DDBJ accession numbers for the nucleotide sequences of the partial Sal9 locus from strain 9 megaplasmid and the complete chromosomally located Sal9 locus of strain JIM8780 are DQ889747 and GQ857551, respectively.

An indirect application of lantibiotics stems from their production by some probiotic bacteria (Burton *et al.*, 2006a). For example, it has been observed that the natural occurrence in the human oral cavity of *Streptococcus salivarius* populations producing bacteriocin-like inhibitory substances (BLIS) correlates with decreased rates of acquisition of the pathogen *Streptococcus pyogenes* (Dierksen & Tagg, 2000; Fantinato *et al.*, 1999). *S. salivarius* K12, the first widely used oral probiotic, has been shown to produce two lantibiotics, salivaricin A2 (SalA2) and salivaricin B (SalB) (Hyink *et al.*, 2007). Other lantibiotic loci have also been identified in *S. salivarius*, including those encoding (a) salivaricin A (SalA) (Ross *et al.*, 1993) and its natural variants salivaricin A3, salivaricin A4 and salivaricin A5 (Wescombe *et al.*, 2006b), (b) salivaricin G32, which differs from the *S. pyogenes* lantibiotic SA-FF22 propeptide by just one amino acid (Wescombe, 2002), and (c) streptin, which is also commonly produced by *S. pyogenes* strains (Wescombe *et al.*, 2006a). The SalA cluster of lantibiotics and streptin have all been shown to act as auto-inducer peptides – being recognized by specific

two-component sensor-kinase/response-regulator systems, which serve to upregulate transcription of the lantibiotic structural gene and thereby effect increased lantibiotic production (Upton *et al.*, 2001; Wescombe & Tagg, 2003).

In our initial studies of *S. salivarius* BLIS, strain 9 was one of six prototype inhibitory strains identified (Dempster & Tagg, 1982). The inhibitory activity of strain 9 was shown to be due, at least in part, to the production of SalA4 (a natural variant of SalA) (Wescombe *et al.*, 2006b). However, it soon became clear that SalA4 does not account for the entire inhibitory activity of strain 9. The present study reports the genetic basis for and peptide identity of salivaricin 9 (Sal9), a novel lantibiotic, the genetic locus of which in *S. salivarius* can be either chromosomal or megaplasmid-located.

METHODS

Bacterial strains and culture conditions. All strains were propagated on Columbia blood agar base (Difco) supplemented with 5 % human blood (BaCa) by incubation in 5 % CO₂ in air at 37 °C. Other media included M17 (Difco) supplemented with 0.5 % sucrose, Todd-Hewitt broth (THB) (Difco) and THB supplemented with CaCO₃ (4 µmol l⁻¹) and glucose (0.1 %, w/v) (THBCaGlu). The principal test strains used in the present study were *S. salivarius* strain 9 (one of the originally described *S. salivarius* bacteriocin producer strains) (Dempster & Tagg, 1982), JIM8780 (Delorme *et al.*, 2007) and M18 (also known as Mia) (Wescombe *et al.*, 2006a). Other details of the strains are listed in Tables 1 and 2.

Deferred antagonism assays. The method of deferred antagonism originally described by Tagg & Bannister (1979) was used either to determine the producer-type (P-type) of BLIS activity of the test strains, or to compare the relative susceptibilities of different bacterial strains to the BLIS activities produced in agar media and to determine the activity

Table 1. Distribution of the Sal9 and SalA structural genes in *S. salivarius* strains of different BLIS P-type designations

<i>S. salivarius</i> strain	Presence of salivaricin gene		P-type	Strain source/reference
	<i>sivA</i>	<i>sala</i>		
193	—	—	777	Wescombe <i>et al.</i> (2006a)
20P3	—	+	677	Ross <i>et al.</i> (1993)
220	+	—	634	J. R. Tagg
36	—	—	777	Dempster & Tagg (1982)
36P [−] *	—	—	000	J. R. Tagg
5	+	+	677	Dempster & Tagg (1982)
6	—	+	777	Dempster & Tagg (1982)
9	+	+	676	Dempster & Tagg (1982)
9P [−] *	—	—	400	This study
DC135B	+	+	677	J. R. Tagg
DC156A	+	—	636	J. R. Tagg
GR	—	—	677	J. R. Tagg
H7f	—	+	677	J. R. Tagg
H21	—	+	777	Hyink <i>et al.</i> (2007)
H25	—	+	777	J. R. Tagg
JH	—	+	777	Tompkins & Tagg (1989)
JIM8777	—	—	226	Delorme <i>et al.</i> (2007)
JIM8780	+	—	636	Delorme <i>et al.</i> (2007)
JO1	+	—	777	J. R. Tagg
K12	—	+	777	Burton <i>et al.</i> (2006b)
K12P [−] *	—	—	000	Wescombe <i>et al.</i> (2006a)
K30	—	+	777	Hyink <i>et al.</i> (2007)
K8P	+	—	226	J. R. Tagg
M18	+	+	677	J. R. Tagg
M18P [−] *	—	—	000	J. R. Tagg
Min5	+	+	777	Hyink <i>et al.</i> (2007)
MPS	+	+	636	J. R. Tagg
NR	—	—	777	Hyink <i>et al.</i> (2007)
Pirie	—	+	777	J. R. Tagg
Pre18	+	+	636	J. R. Tagg
Strong SA	—	+	777	Hyink <i>et al.</i> (2007)
ToveR	—	+	677	Kelstrup (1981)

*Plasmid-cured derivatives of the parent strain.

Table 2. Deferred antagonism activity of *S. salivarius* strains producing either Sal9 or Sal9 plus other bacteriocins

Indicator strain (no. of strains tested)	Inhibition of indicator by producer strain (bacteriocin/s produced)			Indicator strain source/reference
	9 (SalA, Sal9, others)	JIM8780 (Sal9)	M18 (SalA, Sal9, others)	
<i>Micrococcus luteus</i> T18 - Indicator 1	+	+	+	Tagg & Bannister (1979)
<i>Streptococcus pyogenes</i> FF22 - Indicator 2	+	+	+	Tagg & Bannister (1979)
<i>Streptococcus constellatus</i> T29 - Indicator 3	-	-	-	Tagg & Bannister (1979)
<i>Streptococcus uberis</i> ATCC 27958 - Indicator 4	+	-	+	Tagg & Bannister (1979)
<i>Streptococcus pyogenes</i> 71-679 - Indicator 5	+	+	+	Tagg & Bannister (1979)
<i>Lactococcus lactis</i> T-21 - Indicator 6	+	+	+	Tagg & Bannister (1979)
<i>Streptococcus pyogenes</i> 71-698 - Indicator 7	+	+	+	Tagg & Bannister (1979)
<i>Streptococcus pyogenes</i> W-1 - Indicator 8	+	+	+	Tagg & Bannister (1979)
<i>Streptococcus dysgalactiae</i> subsp. <i>equisimilis</i> T148 - Indicator 9	+	-	+	Tagg & Bannister (1979)
<i>Corynebacterium diphtheriae</i> var. <i>gravis</i>	-	-	+	J. R. Tagg
<i>Enterococcus hirae</i>	+	+	+	J. R. Tagg
<i>Enterococcus hirae</i> lyt 14	-	-	+	J. R. Tagg
Gram-negative bacteria (4)*	-	-	-	J. R. Tagg
<i>Lactobacillus acidophilus</i> NCTC 1899	-	-	-	J. R. Tagg
<i>Lactobacillus casei</i> ATCC 7469	-	-	+	J. R. Tagg
<i>Listeria monocytogenes</i> 10403	-	-	+	J. R. Tagg
<i>Moraxella catarrhalis</i> 4	-	-	-	J. R. Tagg
<i>Streptococcus dysgalactiae</i> 1240662	+	-	+	J. R. Tagg
<i>Streptococcus equisimilis</i> 43	-	-	+	J. R. Tagg
<i>Streptococcus pneumoniae</i> pK2	+	-	+	J. R. Tagg
<i>Streptococcus pyogenes</i> (12)†	+	+	+	J. R. Tagg
<i>Streptococcus salivarius</i> Fen	+	-	+	J. R. Tagg
<i>Streptococcus salivarius</i> Reb	-	-	+	J. R. Tagg
<i>Streptococcus uberis</i> D618	+	+	+	Tagg & Vugler (1986)
<i>Staphylococcus aureus</i> ST821707	+	+	+	J. R. Tagg
<i>Staphylococcus aureus</i> CAMP	-	-	-	J. R. Tagg
<i>Staphylococcus aureus</i> Oxford	-	-	-	J. R. Tagg
<i>Streptococcus agalactiae</i> P3	-	-	+	J. R. Tagg
<i>Streptococcus pneumoniae</i> 44	-	-	+	J. R. Tagg

**Neisseria meningitidis*, *Haemophilus influenzae* 29, *H. influenzae* 30, *H. influenzae* 33.

†M-serotypes: M4, M15, M23, M37, M52, M53, M60, M71, M72, M74, M79, M81.

spectrum of Sal9 producers. Briefly, the test strain was inoculated diametrically across the surface of the BaCa medium as a 1 cm-wide streak. After incubation, the test strain growth was removed using a glass slide and the surface of the agar sterilized by exposure to chloroform vapour for 30 min. The plate was then aired for 15 min before 18 h THB cultures of the indicator strains were inoculated across the line of the original producer growth. The plates were then incubated as before for 24 h and examined for zones of interference with the indicator growth. In order to establish the P-type designation, the inhibitory activity against the nine standard indicators was recorded in code form (the P-type) by considering the indicators as three triplets (i.e. I1, I2, I3; I4, I5, I6; and I7, I8, I9). Inhibition of the first member of a triplet was given a score of 4, the second a score of 2, and the third a score of 1. Absence of inhibitor action against an indicator was scored as 0. The P-type code was then recorded as a sequence of three numbers representing the sum of each triplet. All tests were performed in duplicate, and further testing was undertaken until consistency of the inhibition patterns was obtained.

Spot inhibitory assay. To determine inhibitory activity in liquid preparations a 20 µl drop of each preparation was placed on the surface

of a dry BaCa plate, allowed to dry, and the agar surface sterilized with chloroform vapour for 30 min. Following airing for 30 min the indicator organism [usually *Micrococcus luteus* T-18 (indicator I1)] was applied as a lawn by charging a sterile swab with an 18 h THB culture and applying it evenly over the surface of the agar. The titre [in arbitrary units (AU) ml⁻¹] of the inhibitory activity in the original test preparation was defined as the reciprocal of the highest dilution of a doubling dilution series to inhibit the growth of the indicator lawn.

PCR amplification of *siv* and *sal* genes. The nucleotide sequences of several different examples of each of the lantibiotic modifying genes (i.e. *lanM*, *lanB* and *lanC*) have previously (Hyink *et al.*, 2005; Wirawan *et al.*, 2006) been used to design the following degenerate PCR primer sets: *LanM*-fwd, 5'-ATGCWAGWYWTGCWCATGG-3', and *LanM*-rev, 5'-CCTAATGAACCRTRRYAYCA-3'; *LanB*-fwd, 5'-TATGATCGAGAARYAKAWAGATATGG-3', and *LanB*-rev, 5'-TTATTATCAIATGIAYDAWACT-3'; *LanC*-fwd, 5'-TAATTTAGG-ATWISYIMAYGG-3', and *LanC*-rev, 5'-ACCWGKIIICCRTRRCA-CCA-3'. These primers were utilized in the present study to amplify putative lantibiotic loci in strain 9. All amplifications were carried out

using Hotmaster *Taq* polymerase (Eppendorf). Typical PCRs for the degenerate primer sets included 41.5 µl PCR-quality water (Eppendorf), 5 µl 10× Buffer (Hotmaster, Eppendorf), 1 µl nucleotide mix (Roche), 1 µl each of the appropriate forward and reverse primers (primer stocks at 0.1 ng µl⁻¹ for the lantibiotic degenerate primers or 0.01 ng µl⁻¹ for standard PCR) and 0.5 µl *Taq* (Hotmaster, 5 U µl⁻¹). An initial denaturation at 95 °C was carried out for 2 min followed by 30 cycles of 95 °C for 30 s, annealing at 40 °C (for the degenerate lantibiotic primers) or 55 °C (for *sivA* or *salA* amplification) for 30 s and elongation for 1 min at 65 °C. Two per cent agarose gels were run to analyse all PCR products. For the 'universal' lantibiotic amplifications, DNA bands that were of similar size to the control reactions were gel extracted (Qiagen gel extraction kit) and cloned into pGEM-T (Promega) according to the manufacturer's instructions, then sequenced. Inverse PCR techniques were used to amplify the regions outside the modification gene and to obtain sequence for the lantibiotic structural gene(s) (Triglia *et al.*, 1988).

Identification of the locus encoding Sal9. When the draft genome sequence of *S. salivarius* strain JIM8780 (P. Renault, unpublished data) was examined for *sivA* homologues, a locus containing eight genes, including *sivA*, was identified. The locus was characterized by a combination of BLASTN, BLASTX and BLASTP searches against the GenBank database using search engines at the National Center for Biotechnology Information (NCBI; <http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Megaplasmid detection using pulsed-field gel electrophoresis (PFGE). Using previously described methods (Wescombe *et al.*, 2006a), PFGE was used to detect the megaplasmid DNA content of the test strains 9, K12 and JH (PFGE examination of strain JIM8780 failed repeatedly due to apparent autodigestion of the DNA). One millilitre of 18 h Todd-Hewitt Broth (THB) cultures of the test strains was used to inoculate 20 ml THB cultures, which in turn were incubated at 37 °C in 5% CO₂ in air until reaching an optical density (650 nm) of 0.5. The cells were then embedded in low-melting-point agarose (SeaPlaque, FMC BioProducts) and lysed using 40 mg lysozyme ml⁻¹ (Roche), followed by treatment with proteinase K (1 mg ml⁻¹) (Sigma). The proteinase K was removed by six 1 h TE (10 mM Tris, 1 mM EDTA, pH 7.5) washes with shaking (120 r.p.m.) at room temperature. After digestion with *Sma*I, the DNA was separated using a CHEF-DR III Pulsed Field Electrophoresis System (Bio-Rad) over 20–23 h in a 1% agarose gel (Pulsed Field Certified Agarose, Bio-Rad). An initial pulse time of 6 s and final of 18 s were used. The included angle (change in orientation of the field) was 120°, and the gel was run at 4.5 V cm⁻¹ with the buffer

maintained at 14 °C. Gels were stained with ethidium bromide (5 mg ml⁻¹) and examined by UV transillumination.

Localization of the *siv* locus using Southern blotting and DNA probing. DNA from PFGE was transferred to Hybond-N+ nylon membranes (Amersham Biosciences) using a VACUGENE 2016 vacuum blotting unit (Pharmacia LKB Biotechnology) and fixed to the membrane by exposure to 0.4 M NaOH for 20 min. Probes for hybridization were generated using (a) the *sivA* primers *sivF* (5'-AAAAAGGCGCTTCTATATCCATGA-3') and *sivR* (5'-ATCTTT-ACCTCAAACCTTTTAAGTCCATT-3') and (b) the *salA* primers *salAF* (5'-GATATTTTGAACAATGCTATCGAAG-3') and *salAR* (5'-ACTAATAGAAGTATCTAGTATGTCG-3'). The amplification parameters were 30 cycles of 95 °C for 30 s, annealing at 55 °C for 30 s and elongation of 1 min at 65 °C. Radiolabelling was done by use of the Ready-To-Go DNA Labelling Beads (dCTP) kit (Amersham Biosciences). These probes were used to localize the lantibiotic loci on PFGE blots by autoradiography.

Purification of Sal9. *S. salivarius* strain 9 typically produces only very small quantities of bacteriocin when grown in liquid cultures. To overcome this problem, a method was devised to obtain enhanced (induced) bacteriocin production by supplementing the growing strain 9 cultures with pre-formed Sal9. Inducer preparations of Sal9 were prepared by growing *S. salivarius* strain 9 lawn cultures for 18 h at 37 °C in 5% CO₂ in air on M17 agar supplemented with 0.5% sucrose. The agar culture was then frozen at -70 °C and subsequently thawed. The exudate fluid was collected and assayed for inhibitory activity against *M. luteus* using the 20 µl spot assay. One hundred microlitres of preparations of titre greater than 2 was added per 10 ml of THB freshly inoculated (1%, v/v) from an 18 h THB culture of strain 9. This achieved enhanced (induced) production of Sal9 during the subsequent growth at 37 °C for 18 h in 5% CO₂ in air. It was found that when the cells from each 10 ml culture were harvested by centrifugation (10 300 g for 10 min), resuspended in 1 ml 95% methanol containing 25 mmol HCl l⁻¹ and incubated for 18 h at 4 °C, a relatively pure bacteriocin preparation could be obtained when compared to that from the culture supernatants. After removing the cells by centrifugation, the bacteriocin-containing supernatant was concentrated 10-fold by evaporation under vacuum using a Speedvac centrifuge (Eppendorf), then fractionated by HPLC on a C18 reversed-phase column (Phenomenex Jupiter C18 column, 5 µm, 300 Å, 250 × 4.6 mm) using a gradient of 20 to 50% acetonitrile over 50 min and a flow rate of 1 ml min⁻¹. Fractions having inhibitory activity against *M. luteus* were further analysed by matrix-assisted

Fig. 1. Schematic of the Sal9 locus in *S. salivarius* strain JIM8780 and the corresponding insertion region in the chromosome of JIM8777. The region for which DNA sequence was obtained from strain 9 is shown by the dashed line. The black arrows represent truncated transposase genes and dashed oblongs are traces of genes of unknown functions. Proposed functions for each gene in the Sal9 locus are as follows: *sivK*, putative sensor kinase; *sivR*, putative response regulator; *sivA*, Sal9 precursor peptide; *sivM*, putative lantibiotic modification enzyme; *sivT*, putative ABC transporter involved in the export of Sal9 and concomitant cleavage of its leader peptide; and *sivFEG*, encoding lantibiotic self-immunity.

Fig. 2. (a, b) Alignment of (a) the leader sequences and (b) the propeptide sequences of known lantibiotics belonging to the SA-FF22/lacticin 481 family with Sal9. Shaded residues indicate amino acids in common with Sal9. (c) Schematic of the putative bridging structure of Sal9. Modified residues are shaded: b, dihydrobutyryne; b-S-a, 3-methylanthionine.

laser desorption-time of flight (MALDI-TOF) mass spectrometry (MS) at the Protein Microchemistry Facility, Department of Biochemistry, University of Otago (Hubbard & McHugh, 1996).

Induction of BLIS production in *S. salivarius* strains 9, JIM8780 and 20P3. The specificity of the bacteriocin-inducing activities present in crude extracts of *S. salivarius* strain 9 cultures and in HPLC-purified peptide fractions having anti-*M. luteus* activity was assessed using previously described methods (Wescombe *et al.*, 2006b). Briefly, 10 ml THB cultures of *S. salivarius* strains 9, JIM8780 and 20P3 were grown for 18 h at 37 °C in 5 % CO₂ in air. These cultures were centrifuged (15 300 g for 1 min) and the pellets washed three times in 10 ml volumes of 0.85 % NaCl (w/v) to reduce the levels of cell surface-associated lantibiotic/signal peptide. The cells were then resuspended in 10 ml 0.85 % NaCl. THBCaGlu (20 ml) was inoculated with 100 µl of washed cells, and 180 µl aliquots of this cell suspension were dispensed into wells in a microtitre plate. Six wells were used for each sample to be tested for SalA- or Sal9-inducing activity. Three wells in each set were designated as controls (i.e. uninduced) and three as tests. To each of the test wells, 10 µl of the test sample was added. After 18 h incubation at 37 °C in 5 % CO₂ in air, 10 µl of test sample was added to each of the three control wells. Samples (50 µl) from each of the test and control wells were then tested for inhibitory activity against indicator I1 using the agar well diffusion assay as described previously (Wescombe *et al.*, 2006b). Induction of SalA or Sal9 production was demonstrated by the appearance of inhibitory zones surrounding the test wells, but not the corresponding control wells.

RESULTS AND DISCUSSION

Identification of a novel lantibiotic locus in *S. salivarius* strain 9

In the present study, PCR using a set of *lanM*-specific primers first detected the locus of a putative new member of the streptococcal A-FF22/lacticin 481 lantibiotic family (Dufour *et al.*, 2007) in *S. salivarius* strain 9. Inverse PCR then extended the sequence beyond the *lanM* gene to identify 6277 bp encoding the corresponding structural gene and regulatory elements of the locus (Fig. 1). The Sal9 propeptide is significantly different from its closest homologue, SA-FF22, having only 46 % identity at the amino acid level (11 out of 24 residues identical) (Fig. 2b). Despite these differences, the thioether bridges within the propeptide are predicted to be conserved, indicating that they share similar molecular conformations (Fig. 2c). Interestingly, the predicted propeptide of Sal9 contains no serine, making it the first member of the SA-FF22/lacticin 481 lantibiotic family to lack serine (Dufour *et al.*, 2007). A further distinguishing feature of Sal9 is that the predicted dehydro- residue (in this lantibiotic family characteristically situated directly adjacent to the C-terminal cysteine) is in Sal9 located 5 residues N-terminal to the cysteines (Fig. 2b). While it is not at present

Fig. 3. Analysis of megaplasmid content of *S. salivarius* strains K12, 9 and JH. (a) PFGE of *S. salivarius* strains K12 (lanes 2 and 3), 9 (lanes 4 and 5) and JH (lanes 6 and 7) total DNA. Lanes 2, 4 and 6 are uncut DNA while lanes 3, 5 and 7 are *SmaI* digests. (b) Autoradiograph of PFGE gel in (a), probed with radiolabelled SalA DNA. (c) Autoradiograph of PFGE gel in (a), probed with radiolabelled Sal9 DNA. (d) Overexposed and inverted image of the boxed area in (a) revealing the plasmid bands in the uncut lanes (2, 4 and 6). Arrows indicate plasmid bands.

clear whether this may affect the specific activity of the molecule, its novelty would encourage a site-directed mutagenesis approach to shed more light on the significance of this difference.

The DNA sequence obtained from strain 9 PCR products allowed identification of the entire *Sal9* locus in the unpublished genome sequence of *S. salivarius* strain JIM8780. The *Sal9* nucleotide sequences of strain 9 and strain JIM8780 share 98.9% identity, with the predicted translation products having a mean identity of 98% (range 97–99%). The DNA sequence of the *Sal9* locus includes: (a) *sivK*, encoding a putative sensor kinase, and *sivR*, the corresponding putative response regulator, which together are predicted to be involved in the regulation of *Sal9* biosynthesis (Hyink *et al.*, 2007; McLaughlin *et al.*, 1999; Upton *et al.*, 2001); (b) *sivA*, which encodes the *Sal9* precursor peptide; (c) *sivM*, the gene encoding the putative lantibiotic modification enzyme; (d) *sivT*, having close homology with *scnT* (the SA-FF22 ABC transporter) and thought likely to encode an ABC transporter involved in the export of *Sal9* and concomitant cleavage of its leader peptide; and (e) *sivFEG*, which corresponds to gene clusters typically involved in lantibiotic self-immunity (Fig. 1). There is one predicted amino acid difference at position 10 of the *Sal9* leader sequence, with serine present in strain JIM8780 and alanine in strain 9. Because this sequence is not highly conserved in other lantibiotic leaders (Fig. 2a), it is not expected that this would result in a significant difference in either the peptide's post-translational processing or its transport from the cell.

The locus resembles that of a number of other lantibiotics of the lactacin 481 family, but is most similar to SA-FF22/macedocin loci (McLaughlin *et al.*, 1999; Papadelli *et al.*, 2007). The degree of identity at the amino acid level of the translated gene products of the *Sal9* locus with the equivalent gene products from SA-FF22 was determined by BLASTX analysis as: *SivK/ScnK*, 46%; *SivR/ScnR*, 68%; *SivA/ScnA*, 65%; *SivM/ScnM*, 48%; *SivT/ScnT*, 60%; *SivF/ScnF*, 67%; *SivE/ScnE*, 35%; and *SivG/ScnG*, 45%.

Megaplasmid localization of the *Sal9* and *SalA* lantibiotic loci in *S. salivarius* strain 9

The *Sal9* and *SalA* loci were localized to an approximately 170 kbp megaplasmid in strain 9 (Fig. 3), bringing the number of megaplasmid-located lantibiotic loci in *S. salivarius* to five (Wescombe *et al.*, 2006a). Linear forms of megaplasmids were visualized in the undigested total DNA (lanes 2, 4 and 6 in Fig. 3d) as single faint bands. Only linear forms of megaplasmids migrate at rates that allow size determination by comparison with linear markers. Closed circular supercoiled forms move very slowly, and relaxed and nicked open circular forms remain trapped in the sample wells (Barton *et al.*, 1995). Southern analysis of the PFGE with DIG-labelled DNA probes encompassing *sivA* or *salA* did

not detect *sivA* in either the chromosome or the 190 kbp and 220 kbp plasmids harboured by the bacteriocinogenic *S. salivarius* strains K12 and JH, respectively (Fig. 3c). In all three strains, however, each megaplasmid was shown to be probe-positive for *SalA* by Southern blotting (Fig. 3b). The restriction enzyme used (*SmaI*) appeared to linearize the plasmids (i.e. cut the plasmid only once), possibly due to the low G+C content of *S. salivarius* and the fact that the recognition sequence of *SmaI* is CCCGGG, making it a rare cutter of *S. salivarius* DNA – for example, *SmaI* has only 17 recognition sites in strain JIM8780. This resulted in an increased release of megaplasmid from the agarose plug, which was visualized as a brighter DNA band. These bands were identified as plasmid during the Southern

Fig. 4. MALDI-TOF MS analysis of fractions A and B from HPLC fractionation of strain 9 methanol extracts from spiked culture cells. The mass assigned to the major peak in each fraction is shown. Fraction A corresponds to the mass predicted for *Sal9* while the mass in fraction B is equivalent to that of *SalA4* previously characterized from *S. salivarius* strain 9.

analysis because they were the same size as the hybridization band in the uncut DNA lanes as indicated by the arrows in lanes 3, 5 and 7 in Fig. 3(b). Further confirmation of the plasmid location of the Sal9 locus was obtained through curing strain 9 of its megaplasmid resulting in strain 9P⁻, which is both *sivA*- and *salA*-negative (Table 1). By contrast, the Sal9 locus is chromosomally located in strain JIM8780 (as determined by complete genome sequencing), this being to our knowledge the first established example of a lantibiotic locus being other than plasmid-borne in *S. salivarius*. Flanking the Sal9 locus in strain JIM8780 are incomplete transposase genes which may have provided a mechanism for the movement of the locus within *S. salivarius* and its insertion between the chromosomally located *gtfD* and *gtfI* genes of the glucosyltransferase locus in strain JIM8780 (Fig. 1).

Distribution of the Sal9 locus

A PCR screen, using the primer pairs *sivAF/sivAR* (to amplify *sivA*) and *salAF/salAR* (to amplify *salA*), was applied to a selection of *S. salivarius* strains known to produce various BLIS activities (Table 1). Twelve (43 %) of the 28 individual strains were positive for *sivA*, indicating that the Sal9 locus is widespread in BLIS-positive *S. salivarius*. In addition, 18 (64 %) of the 28 individual strains were positive for *salA* and 7 (25 %) of the strains had both *sivA* and *salA*. Since *salA* is widespread in *S. pyogenes* (Simpson *et al.*, 1995) and *sboA* (encoding salivaricin B) is present in some *Streptococcus mitis* strains (Hyink *et al.*, 2007), 116 *S. pyogenes* of different M-types and 71 *S. mitis* strains were screened by Southern hybridization for *sivA*. None of the strains tested was probe-positive for *sivA*, indicating that the Sal9 locus may not be commonly (if at all) present in these two oral streptococcal species.

Partial purification of Sal9

Sal9 was purified from methanol extracts of cells of *S. salivarius* strains 9 and JIM8780 by C18 reversed-phase HPLC. Two separated fractions (A and B) (results not shown) from the strain 9 extract had inhibitory activity against *M. luteus* and following MALDI-TOF MS analysis the mass of fraction A (2560.6 Da) matched that predicted for Sal9 (Fig. 4a), whereas the mass of fraction B (2341.6 Da) corresponded to that of SalA4 (Fig. 4b) (Wescombe *et al.*, 2006b). A single inhibitory fraction was obtained from C18 reversed-phase chromatography of strain JIM8780 extract and this also contained a MALDI-TOF MS mass of 2560.6 Da.

Activity spectra of Sal9-producing strains

S. salivarius strains 9, M18 (also known as Mia) (Wescombe *et al.*, 2006a) and JIM8780, all of which contain *sivA*, were tested for inhibitory activity against an extended set of both Gram-positive and Gram-negative indicator bacteria (Table 2). Inhibitory activity attributable to Sal9 alone appears to be produced by strain JIM8780 and gives the characteristic P-type of 636 (inhibition of 6/9 standard indicators). Both strain 9 and strain M18 also produce variants of SalA and some as yet uncharacterized additional inhibitory agent(s). The inhibitory spectrum of JIM8780 includes strains of *S. pyogenes*, *Lactococcus lactis*, *Enterococcus hirae*, *Staphylococcus aureus*, *Streptococcus uberis* and *Streptococcus salivarius*, indicating that Sal9 production by *S. salivarius* probiotics such as M18 could potentially contribute more widely to infection control. Sal9 may be especially beneficial when produced by strains also producing SalA and SalB, since the action of multiple bacteriocins serves to reduce the risk of resistance development to the individual bacteriocins in the target

Table 3. Comparative activity of the lantibiotics SalA, Sal9 and nisin A against selected indicator bacteria

Indicator strain	Lantibiotic phenotype	Inhibition of indicator strain by		
		JIM8780 (Sal9)	20P3 (SalA)	1404 (nisin A)
<i>L. lactis</i> A5	Nisin Z	—	+	—
<i>L. lactis</i> 1404	Nisin A	—	+	—
<i>L. lactis</i> I6		+	+	+
<i>S. salivarius</i> H25	SalA, SalB	—	—	+
<i>S. salivarius</i> H7f	SalA	+	—	+
<i>S. salivarius</i> JIM8780	Sal9	—	+	+
<i>S. salivarius</i> 9	SalA, Sal9	—	—	+
<i>S. salivarius</i> 20P3	SalA	+	—	+
<i>S. salivarius</i> K12	SalA, SalB	—	—	+
<i>S. salivarius</i> K12 P ⁻ *		+	+	+
<i>S. salivarius</i> M18	SalA, Sal9	+/-†	—	+
<i>S. salivarius</i> M18 P ⁻ *		+	+	+

*Plasmid-cured derivatives of the parent strain.

†Weak inhibition, with many resistant colonies growing in the inhibition zone.

Table 4. Induction of inhibitor production by *S. salivarius* strains 9, JIM8780 and 20P3 using crude preparations and purified inhibitor-positive fractions from each strain

Inhibitor-positive preparation tested for inducing activity	Lantibiotic peptide(s) in preparation	Preparation induces inhibitor production in <i>S. salivarius</i> strain		
		9	20P3	JIM8780
Strain 9 crude	SalA and Sal9	Yes	Yes	Yes
Strain 9 Fraction A	Sal9	Yes	No	NT
Strain 9 Fraction B	SalA	Yes	Yes	NT
Strain 20P3 crude	SalA	Yes	Yes	No
Strain JIM8780 crude	Sal9	Yes	No	Yes
Strain JIM8780 purified inhibitor	Sal9	Yes	No	Yes

NT, Not tested.

organisms. Since none of the nine standard indicator strains conventionally used in this laboratory for P-typing of BLIS-positive streptococci appeared specifically to detect Sal9, a separate set of indicator strains was tested for their relative sensitivities to Sal9 and SalA (Table 3). The strains *S. salivarius* H7f and *S. salivarius* 20P3 (both of which are SalA positive) were inhibited by *S. salivarius* JIM8780 but not by *S. salivarius* 20P3, and thus either of these strains could be used to specifically and selectively detect Sal9 (but not SalA) activity.

Specificity of Sal 9 induction

Since other lantibiotics such as SalA have been found to specifically induce their own production (Kuipers *et al.*, 1995; Upton *et al.*, 2001), crude inhibitor-positive culture extracts from *S. salivarius* strains 9, JIM8780 and 20P3 (a known SalA producer), as well as inhibitor-positive fractions purified from the crude extracts of strains 9 and JIM8780, were analysed for their ability to induce inhibitor production by *S. salivarius* strains 9, JIM8780 and 20P3 (Table 4). Whereas the crude extract from strain 9 was found to induce inhibitor production by both strain 9 and strain 20P3, the purified Sal9 peptide in fraction A only induced inhibitor production in strain 9, while the fraction B peptide (i.e. SalA) induced inhibitor production in both strains. It must be noted, however, that while the Sal9 preparation used appeared to be pure, there have been reports of induction peptides co-purifying with bacteriocins (Diep *et al.*, 1995) and therefore it is possible that a low level of a different molecule may be responsible for the induction observed. Both the crude extract and the purified inhibitory fraction from strain JIM8780 only induced inhibitor production in strains JIM8780 and 9, whereas the crude extract from strain 20P3 induced inhibitor production in both strain 20P3 and strain 9. These results indicate that the predicted molecular conformational differences between the Sal9 and SalA peptides are significant and Sal9 falls outside the requirements for specific recognition by the sensor kinase SalK.

Concluding remarks

It seems evident from their ubiquitous presence in *S. salivarius* that lantibiotics must play an important ecological role within the microbiota of oral mucosal surfaces. Sal9 with its inhibitory activity against *S. pyogenes* may prove to be an important lantibiotic in the oral probiotic arena. For example, *S. salivarius* strain M18 (marketed in the USA) was shown in the present study to encode Sal9 as potentially one of the mechanisms through which it effects improved oral health. In addition, the availability of technology for transfer of *S. salivarius* megaplastids to plasmid-free *S. salivarius* (Wescombe *et al.*, 2006a) now facilitates the tailor-making of designer probiotic strains containing desirable combinations of BLIS armoury, colonization efficiency and immune stimulatory capability. The probiotic potential of *S. salivarius* should continue to maintain research interest in the further characterization of novel inhibitors produced by this species.

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