Isolation and partial characterization of the *Streptococcus mutans* type All lantibiotic mutacin K8

Cynthia L. Robson,¹ Philip A. Wescombe,² Nikolai A. Klesse² and John R. Tagg¹

Correspondence
Philip A. Wescombe
philip.wescombe@blis.co.nz

¹Department of Microbiology and Immunology, University of Otago, PO Box 56, Dunedin, New Zealand

²BLIS Technologies Ltd, Centre for Innovation, PO Box 56, Dunedin, New Zealand

Streptococcus mutans strain K8 was shown to produce a newly identified type All lantibiotic, mutacin K8. The mutacin K8-encoding *muk* locus consists of 13 ORFs, three of which (*mukA1*, *A2* and *A3*) have close homology to *scnA*, the structural gene encoding the *Streptococcus pyogenes* lantibiotic SA-FF22, and another (*mukA'*) resembles *scnA'*, an ORF in the SA-FF22 locus that has no currently assigned function. Inactivation of the *muk* locus indicated that mutacin K8 is responsible for most of the inhibitory activity produced by strain K8 in deferred antagonism tests on Columbia blood agar base supplemented with 5% human blood and 0.1% CaCO₃. By contrast, on tryptic soy agar plus 2% yeast extract and 0.5% CaCO₃ most of the inhibitory activity of strain K8 appeared to be attributable either to mutacin IV or to some other inhibitory peptide(s) exported by the mutacin IV transporter *nImT*. An inhibitory peptide purified from a derivative of strain K8 in which *nImT* had been inactivated had a mass of 2734 Da and an N-terminal sequence identical to the predicted propeptide translation products of *mukA1* and *mukA3*. The *muk* locus may be widely distributed in *S. mutans*, since 9 (35%) of 26 strains tested contained at least part of the locus. In the genome sequence of strain UA159 the *muk* locus is incomplete, the sole residual components being the ORFs encoding the putative two-component regulatory system *mukR* (SMU.1815) and *mukK* (SMU.1814), followed by two transposases (SMU.1813 and SMU.1812) and then the ORFs *mukF* (SMU.1811), *mukE* (SMU.1810) and *mukG* (SMU.1809), thought to encode putative immunity peptides. Strains such as UA159 having incomplete loci did not produce detectable levels of mutacin K8.

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INTRODUCTION

The first streptococcal bacteriocin to be isolated and partially characterized was streptococcin A-FF22 (SA-FF22), initially referred to as streptocin A (Tagg *et al.*, 1973b). Partially purified SA-FF22 inhibited a variety of Gram-positive bacteria (Jack & Tagg, 1991; Tagg *et al.*, 1973a) and when tested against nine standard indicator bacteria in a streptococcal bacteriocin fingerprinting scheme the producer strain *Streptococcus pyogenes* FF22 (serotype M52) produced a pattern of inhibition referred to as bacteriocin production (P)-type 436 (Tagg & Bannister, 1979). SA-FF22 was subsequently shown to be a 2794.98 Da, nisin-like lantibiotic (Tagg & Wannamaker, 1978; Jack & Tagg, 1991). Following the cloning of the SA-FF22 structural gene (*scnA*) in strain FF22, closely homologous genes were

shown to be present relatively frequently in serotype M49 *S. pyogenes*, although these strains typically contained two copies (*scnA* and *scnA1*) of the SA-FF22 structural gene (Hynes *et al.*, 1994). Subsequent sequencing of the entire SA-FF22 locus in strain FF22 demonstrated that *scnA'*, a markedly variant form of *scnA*, lies 203 bp downstream of *scnA* (McLaughlin *et al.*, 1999). Although *scnA'* was shown to be transcribed, no evidence for expression of a biologically active product has been reported and thus no function has yet been assigned (McLaughlin *et al.*, 1999).

Strain EB1 is a derivative of strain FF22 that no longer produces SA-FF22 and that also has specifically enhanced sensitivity (i.e. loss of putative immunity) to SA-FF22 (Tagg & Wannamaker, 1976). Our recent observation that several strains of streptococcal species other than *S. pyogenes* also exhibit relatively stronger inhibitory activity against strain EB1 than strain FF22 raised the prospect that bacteriocins produced by these strains may at least functionally resemble SA-FF22. The first lantibiotic shown to have homologues

Abbreviation: BLIS, bacteriocin-like inhibitory substance.

The GenBank/EMBL/DDBJ accession number for the sequence of the partial mutacin K8 locus from *S. mutans* strain K8 is EF060238.

produced by strains of more than one streptococcal species was salivaricin A (Simpson *et al.*, 1995; Upton *et al.*, 2001; Wescombe *et al.*, 2006). Subsequently, the *Streptococcus uberis* lantibiotic nisin U has also been found to be produced by a variety of streptococcal species (Wirawan *et al.*, 2006). Here we report that *Streptococcus mutans* strain K8 produces mutacin K8, a lantibiotic strongly homologous to SA-FF22.

METHODS

Bacterial strains and culture media. The *S. mutans* strains used in the present study are listed in Table 1. Bacterial strains used as indicators are listed in Table 2. The indicators I1 to I9 are a set routinely used in this laboratory to detect bacteriocin activity (Tagg & Bannister, 1979). Culture growth was on either Columbia agar base (Difco) supplemented with 0.1% CaCO₃ and 5% human blood (BaCa) or Bacto tryptic soy broth (Becton Dickinson) supplemented with 2% (w/v) Bacto yeast extract, 1.5% (w/v) bacteriological agar (Scientific Supplies) and 0.25% (w/v) CaCO₃ (TsyCa) for 18 h at 37 °C in 5% CO₂ in air. *S. mutans* transformants were selected on brain heart infusion agar supplemented with 0.5% yeast extract and 2 µg erythromycin ml⁻¹. The medium used for production of mutacin K8 was THB agar, which comprised Todd–Hewitt broth (Difco) supplemented with 1% (w/v) bacteriological agar, aliquoted as 20 ml volumes in Petri dishes.

Table 1. *S. mutans* strains tested for mutacin production and for their content of known mutacin genes

Strain	Species	Source/reference
H7	<i>S. mutans</i>	J. R. Tagg
Ny266	<i>S. mutans</i>	Parrot <i>et al.</i> (1989)
54N	<i>S. mutans</i>	J. R. Tagg
11b	<i>S. mutans</i>	J. R. Tagg
JH1140	<i>S. mutans</i>	Hillman <i>et al.</i> (1998)
K49	<i>S. mutans</i>	J. R. Tagg
K86	<i>S. mutans</i>	J. R. Tagg
31b	<i>S. mutans</i>	J. R. Tagg
K47	<i>S. mutans</i>	J. R. Tagg
H23	<i>S. mutans</i>	J. R. Tagg
K18	<i>S. mutans</i>	Crooks <i>et al.</i> (1987)
M46	<i>S. mutans</i>	J. R. Tagg
4N	<i>S. mutans</i>	J. R. Tagg
K8	<i>S. mutans</i>	Crooks <i>et al.</i> (1987)
D9	<i>S. mutans</i>	J. R. Tagg
K21	<i>S. mutans</i>	Crooks <i>et al.</i> (1987)
G31	<i>S. mutans</i>	J. R. Tagg
K24	<i>S. mutans</i>	J. R. Tagg
M19	<i>S. mutans</i>	J. R. Tagg
UA96	<i>S. mutans</i>	Caufield <i>et al.</i> (1990)
N	<i>S. mutans</i>	Hale <i>et al.</i> (2004)
UA159	<i>S. mutans</i>	Ajdic <i>et al.</i> (2002)
34P	<i>S. mutans</i>	J. R. Tagg
UA140	<i>S. mutans</i>	Qi <i>et al.</i> (2001)
Gs5	<i>S. mutans</i>	J. R. Tagg
K34-1	<i>S. mutans</i>	J. R. Tagg

Deferred antagonism method. The method of deferred antagonism originally described by Tagg & Bannister (1979) was used, either to determine the producer-type (P-type) of bacteriocin-like inhibitory substance (BLIS) activity of the test strains or to compare the relative susceptibilities of different bacterial strains to the BLIS activities produced in agar media. Briefly, the test strain was inoculated on the surface of the agar medium as a 1 cm wide streak across the full diameter of the plate. Following incubation, the visible growth of the test strain was removed using a glass slide, and the surface of the agar was sterilized by exposure to chloroform vapour for 30 min. The plate was then aired for 15 min prior to inoculating 18 h THB cultures of the indicator strains across the line of the original producer growth. The plates were then reincubated for 24 h and examined for zones of interference with the indicator growth. For the purposes of P typing, the inhibitory activity against the nine standard indicators was recorded in code form (the P type) by considering the indicators to be three triplets (i.e. I1, I2, I3; I4, I5, I6; and I7, I8, I9). Inhibition of the first member of an indicator triplet was given a score of 4, that for the second a score of 2, and that for the third a score of 1. No inhibition of an indicator was scored as 0. The complete P-type code was recorded as a sequence of three numbers representing the sum of each triplet. All tests were performed in duplicate, and further testing was undertaken if significant discrepancies were detected in the inhibition patterns obtained.

DNA extraction and PCR amplification. DNA was prepared using a lithium chloride extraction method (Wirawan *et al.*, 2006). All PCR reactions used an Eppendorf Mastercycler thermal cycling apparatus. Elongation times were 1 min at 65 °C for every kb of DNA to be amplified, annealing temperatures as shown in Table 3. Reagents used were HotMaster Taq DNA polymerase (5 U µl⁻¹) and 10× PCR reaction buffer (Eppendorf). Each reaction mix contained 2 µl DNA, 1.5 µl (15 pmol) of each primer, 5 µl 10× buffer, 1 µl nucleotide mix (Eppendorf), 0.5 µl Taq polymerase and sterile deionized water (to a final volume of 50 µl). A set of 26 *S. mutans* strains were screened for known mutacin genes using the following primer pairs (Table 3): mukA upper and mukA lower (mutacin K8); mutI F and mutI R (mutacin I); mutII upper and mutII lower (mutacin II); mutIIIintF and mutIIIintR (mutacin III); mutIVKoF and mutIVKoA (mutacin IV); BHTA DS and BHTA US (Smb); mutN F and mutN R (mutacin N).

For amplification of DNA segments longer than 5 kb, the Expand Long Template PCR system (Roche) incorporating buffer 2 was used according to the manufacturer's recommendations. The PCR programme parameters were 94 °C for 2 min followed by 30 cycles of 94 °C for 10 s, 55 °C for 30 s and 68 °C for 6 min, with an addition of 20 s per cycle after the first 10 cycles. PCR primers used in this study are listed in Table 3.

Inactivation of the mutacin K8 locus and the mutacin IV ABC transporter *nImT*. The mutacin K8 locus was disrupted using a PCR ligation strategy to replace a portion of the *mukM* (5' end) and *mukT* (3' end) genes with the erythromycin resistance determinant *ermAM* (Brehm *et al.*, 1987). Mutacin IV production was eliminated by insertion of *ermAM* into *nImT*, which encodes the ABC transporter that effects export of non-lantibiotic mutacins (Hale *et al.*, 2005a). PCR ligation mutagenesis was based on the Hale *et al.* (2005b) modification of the original Lau *et al.* (2002) procedure. In essence, two sets of primers are utilized to generate each mutant. Primers mutK8P1 and mutK8P2-*EcoRI* amplified the 5' portion of *mukM*, while primers mutK8P3-*PstI* and mutK8P4 were used to amplify the 3' end of *mukT*. The mutK8P2-*EcoRI* and mutK8P3-*PstI* primers incorporate a restriction site to facilitate cloning of the antibiotic marker. PCR amplification utilized HotMaster Taq DNA polymerase, as described above.

Table 2. Bacterial strains used as indicators of bacteriocin activity

Strain	Species	Source/reference
I1	<i>Micrococcus luteus</i>	Tagg & Bannister (1979)
I2	<i>Streptococcus pyogenes</i> M-type 52	Tagg & Bannister (1979)
I3	<i>Streptococcus constellatus</i>	Tagg & Bannister (1979)
I4	<i>Streptococcus uberis</i>	Tagg & Bannister (1979)
I5	<i>S. pyogenes</i> M-type 4	Tagg & Bannister (1979)
I6	<i>Lactococcus lactis</i>	Tagg & Bannister (1979)
I7	<i>S. pyogenes</i> M-type 28	Tagg & Bannister (1979)
I8	<i>S. pyogenes</i> M-type 87	Tagg & Bannister (1979)
I9	<i>Streptococcus dysgalactiae</i>	Tagg & Bannister (1979)
67	<i>S. dysgalactiae</i>	J. R. Tagg
G39	<i>Streptococcus anginosus</i>	J. R. Tagg
K33	<i>S. anginosus</i>	J. R. Tagg
K11	<i>S. anginosus</i>	J. R. Tagg
K24 GAS	<i>S. pyogenes</i>	J. R. Tagg
A5	<i>L. lactis</i>	J. R. Tagg
FF22*	<i>S. pyogenes</i>	Tagg & Wannamaker (1976)
EB1*	<i>S. pyogenes</i>	Tagg & Wannamaker (1976)
ATCC 10556	<i>Streptococcus sanguinis</i>	J. R. Tagg
1506	<i>Streptococcus equi</i>	J. R. Tagg
ATCC 9790	<i>Enterococcus hirae</i>	Cornett <i>et al.</i> (1978)
Lyt 14	<i>E. hirae</i>	Cornett <i>et al.</i> (1978)
C2102	<i>L. lactis</i>	J. R. Tagg

*Strains used both as producers and indicators of bacteriocins.

The PCR products and plasmid pSLER1 [pSL1190 (Pharmacia) containing *ermAM* (Lau *et al.*, 2002)] were digested (18 h at 37 °C) with *Pst*I and *Eco*RI (Roche), following which the products were combined, cleaned using the V-gene PCR clean up kit (V-Gene Biotechnology) and then ligated (16 °C, for 18 h) using T4 DNA ligase (Roche). Using the outer primers mutK8P1 and mutK8P4 the ligated sequence was amplified to increase the yield of the mutagenic construct.

For the inactivation of mutacin IV expression the mutagenic construct was amplified using primers ComADwR and ComAUfP from *S. mutans* UAΔNlmT, an *nlmT* mutant of *S. mutans* strain UA159 (Hale *et al.*, 2005a). Each mutagenic construct was subsequently used to transform *S. mutans* strain K8 using a protocol described previously by Hale *et al.* (2005a). The *ermAM* determinant was always inserted in the same transcriptional orientation as the gene to be mutated, and as *ermAM* lacks a transcription terminator, any downstream polar effects are minimized (Hale *et al.*, 2005a).

Purification of mutacin K8. Lawn cultures were inoculated onto 36 THB agar plates using swabs charged with growth from 18 h THB agar cultures of *S. mutans* strain K8ΔNlmT. Following incubation, the cells were harvested using sterile swabs and resuspended in 30 ml 95 % methanol (pH 2). After 18 h at 4 °C the cells were removed by centrifugation (15300 g, 10 min) and the supernatant assayed for inhibitory activity by spot assay (Wirawan *et al.*, 2006) using *Micrococcus luteus* I1 as the indicator strain. The methanol was removed by Speedvac (Eppendorf) and 400 μl of concentrated extract was applied to an HPLC system fitted with a Phenomenex Jupiter C18 column (5 U, 3000 nm, 250 × 4.6 mm). The flow rate was 1 ml min⁻¹ with an acetonitrile gradient of 0–100 % in 55 min.

Mass spectrometry and N-terminal sequencing. Fractions corresponding to absorbance peaks of purified inhibitory peptides

eluting from the C18 column were submitted to the Protein Microchemistry Facility (Department of Biochemistry, University of Otago) for mass spectrometry and N-terminal sequencing. The masses were determined by MALDI-TOF MS as described by Hubbard & McHugh (1996). The molecular mass of the peptide was calculated by taking the mean (±SE) of the masses from three to five aims. Automated micro-sequencing utilized a pulsed-liquid protein sequencer (Procise 492, Perkin Elmer/Applied Biosystems) (Hubbard *et al.*, 2000).

RESULTS

Screening of *S. mutans* strains for differential inhibitory activity against *S. pyogenes* strains FF22 and EB1

Three strains (K8, K18 and K24) of the set of *S. mutans* tested for SA-FF22-like inhibitory activity displayed relatively greater activity against strain EB1 (*scnA*-negative) than the parent strain FF22 (*scnA*-positive) (data not shown). Strain K8 was selected as the representative inhibitor-positive *S. mutans* for further study.

Sequencing of the mutacin K8 locus from *S. mutans* strain K8

Five ORFs (SMU.1811–1809 and SMU.1815–1814) having close homology to the SA-FF22 immunity genes (*scnF*, *scnE*, and *scnG*) and regulatory genes (*scnR* and *scnK*) respectively are present in the genome of *S. mutans* strain UA159 (Ajdic

Table 3. PCR primers used in this study

Primer	Oligonucleotide sequence (5'–3')*	Accession no./reference	Locus/gene	Annealing temp. (°C)
ComADwR	TTTGCTATTTTCTTAGA	Hale <i>et al.</i> (2005a)	<i>nlmT</i>	50
ComAUpF	CAAAAATCATAGCAATAT	Hale <i>et al.</i> (2005a)	<i>nlmT</i>	50
mutK8P1	GCAGGAATGGCTATAGCTTTAAGTGAGGTT	This study	<i>muk</i> locus	50
mutK8P2- <i>EcoRI</i>	AAAAGAAATTCGTTGCACTAATCTGGCTACTA-ATACACC	This study	<i>muk</i> locus	50
mutK8P3- <i>PstI</i>	ATTACTGCAGGTCTATAGACAGCGAAAACA-AAGGATGA	This study	<i>muk</i> locus	50
mutK8P4	GCAAAGACAACAATGGCTGAGATAAACTG	This study	<i>muk</i> locus	50
ErmInv1	CCAGTTCGCGTTAAATGCCCTTTACCTG	Hale <i>et al.</i> (2005a)	<i>ermAM</i>	50
ErmInv2	CTTACCCGCCATACCACAGATGTTCCAGAT	Hale <i>et al.</i> (2005a)	<i>ermAM</i>	50
scnK	AAACGGACATTATACTACCAATAGCA	This study	<i>mukK</i>	55
scnF	AAAATCATCTTCATCGTTGTTGTC	This study	<i>mukF</i>	55
scnG	CAAACCTCCGAAAATGACATATCTGCTAAG	This study	<i>mukG</i>	55
scnR	CAAAGGACTTGATTGAGGAGGAGATG	This study	<i>mukR</i>	55
mukA upper	GGGCATTCTTAGCCACTTGTGTTCA	This study	<i>muk</i> locus	50
mukA lower	CCATTACCTGCACCACCGATAACTTG	This study	<i>muk</i> locus	50
mutI F	GCAACGGTTATGCTATCGCT	AF238860	Mutacin I/non-coding	50
mutI R	CTGACGGAGGTCTTTCGAGG	AF238860	Mutacin I/ <i>mutA'</i>	50
mutII upper	TGAACAAGTTAAACAGTAACGCAGTAGT	SMU40620	Mutacin II/ <i>mutA</i>	50
mutII lower	ACAGCAAGTGAAAACATGTTGCCATG	SMU40620	Mutacin II/ <i>mutA</i>	50
mutIIIint F	TGTCAAACACACAATTATTAGAAGTCC	AF154675	Mutacin III/ <i>mutA</i>	50
mutIIIint R	GTAACATTTGAACTACCTGTCCTTGC	AF154675	Mutacin III/ <i>mutA</i>	50
mutIVKoF	GGCCCAACGCAAAATCTTGTGGAGATACG	AE014866.1	Mutacin IV/ <i>smu.152</i>	50
mutIVKoA	CAACGTCAGCCTTACATTGAAGAGATGAAGC	AE014866.1	Mutacin IV/ <i>adhE</i>	50
BHTA DS	TCCTACTAACCACATTGACTGACTGAT	DQ145752.1	Smb/non-coding	50
BHTA US	GGGTAGCTGGGTGGGACAAAGGAGAAG	DQ145752.1	Smb/non-coding	50
mutN F	CCGTCAAGCTGCTGATACATTC	Hale <i>et al.</i> (2004)	Mutacin N/ <i>mutN</i>	50
mutN R	CGATATTTAAACCTGCACCGACTACG	Hale <i>et al.</i> (2004)	Mutacin N/ <i>mutN</i>	50
K8scnA upper	GGGCATTCTTAGCCACTTGTGTTCA	This study	Mutacin K8/ <i>mukA1</i>	50
K8scnA lower	CCATTACCTGCACCACCGATAACTTG	This study	Mutacin K8/ <i>mukA2</i>	50
mutIII T	GTTACCACCATATTCATCAACCTTAGA	AF154675	Mutacin III/ <i>mutT</i>	50
mutIII R	CTTTTCGGAATTGGATATGCTTTGA	AF154675	Mutacin III/ <i>mutR</i>	50

*Underlined sequence indicates restriction enzyme recognition site.

et al., 2002). PCR using the primer pair scnK and scnF (designed to amplify the region between SMU.1814 and SMU.1811) was used to screen the *S. mutans* strains K8 and K24 previously mentioned, as well as strain K21, which had relatively less pronounced differential activity against *S. pyogenes* strains EB1 and SA-FF22. Also included were two *S. mutans* strains (UA159 and H7) that did not exhibit this differential inhibitory activity. Two markedly different PCR product sizes were obtained: the larger (10 kb) was from those strains (K8, K21, K24) displaying some differential inhibitory activity against strains FF22 and EB1 and the smaller (2.5 kb) was from the non-differentially inhibitory strains UA159 and H7 (Fig. 1a).

The 10 kb PCR product amplified from strain K8 was partially sequenced and found to encode several ORFs typical of those found in lantibiotic loci. The locus was

designated *muk* for mutacin K8. The schematic diagram of the *muk* locus (Fig. 1b) shows sequence (shaded) obtained directly from strain K8 while that initially derived from the published strain UA159 genome, and then confirmed by PCR also to be present in *S. mutans* strain K8, is unshaded. The sequence of the 6359 bp partial *muk* locus from strain K8 was analysed using the BLASTX algorithm (Altschul *et al.*, 1997). The sequences of the prepeptides MukA1, MukA2 and MukA3 (Fig. 2c) have closest homology to the SA-FF22 and SA-M49 precursor peptides. However, the putative propeptide sequence of MukA2 shares greatest homology (58% identity) with the propeptide components of the lantibiotics Bvi79a (GenBank accession no. AAK32694.1) and RumA (GenBank accession no. AJ276653) produced by *Butyrivibrio fibrisolvens* and *Ruminococcus gnavus* respectively. On the other hand, the MukA1 and MukA3 propeptides have 73% identity to the SA-FF22 propeptide.

Fig. 1. (a) PCR amplification using scnK and scnF primers. Lane 1 is the Generuler 1 kb ladder, lanes 2–5 are *S. mutans* strains H7, K8, K24 and K21 respectively, lane 6 is the negative control (no DNA template) and lane 7 is the positive control *S. mutans* strain UA159. (b) Organization of the mutacin K8 locus (not to scale). The shaded regions are those for which sequence has been derived from strain K8. Unshaded genes are present in strain UA159 and have been shown to be present in strain K8 by PCR. The top row of genes are present in strains UA159 and K8. The biosynthetic genes in the bottom row are present in strain K8, but not UA159. The area deleted during the insertion of *ermAM* to generate strain K8ΔMutK8 is indicated.

The putative mutacin K8 lantibiotic propeptides MukA1 and MukA3 have just eight amino acid differences from the *S. pyogenes* lantibiotic propeptide SA-FF22, with most of the differences occurring near the N-terminus of the propeptide. By comparison, the MukA2 propeptide has 16 amino acid differences (including the absence of the terminal Cys residue) when compared to SA-FF22. The predicted MukA' propeptide (Fig. 2c) differs from the predicted ScnA' propeptide of the SA-FF22 locus at 7 of 24 amino acids.

PCR ligation mutagenesis of the mutacin K8 locus and of *nlmT*, which encodes the ABC transporter of non-lantibiotic bacteriocins such as mutacin IV

The mutacin K8 locus and the non-lantibiotic ABC transporter were separately inactivated in *S. mutans* strain K8 and the resulting effects on inhibitor production were analysed by deferred antagonism on BaCa and TsyCa (Table 4). The inhibitory activity (P-type 100) of K8ΔMutK8 on BaCa was markedly less than the activity (P-type 776) of the parent K8 strain. The P-type 100 (anti-I3) activity appears to be due to either mutacin IV, mutacin N or some other as yet uncharacterized inhibitory peptide

transported by NlmT, since this activity was absent in the transporter mutant strain K8ΔNlmT. On TsyCa medium the production of mutacin K8 by strain K8ΔNlmT appeared relatively less (P-type 410) than that produced on BaCa (P-type 676). By comparison, the production by strain K8ΔMutK8 of mutacin IV, mutacin N and/or other inhibitory peptides transported by NlmT, appeared to be substantially greater on TsyCa (P-type 377) than on BaCa (P-type 100).

The inhibitory spectra of *S. mutans* K8 and its mutant derivatives were compared with those of *S. pyogenes* strains FF22 (streptococcin-producing) and EB1 (not streptococcin-producing) in deferred antagonism tests (Table 5). Indicators that appeared to be inhibited by mutacin K8 (i.e. only inhibited by strain K8 and strain K8ΔNlmT) and not by SA-FF22 were *S. pyogenes* strain FF22 and *Streptococcus dysgalactiae* strain 67. By contrast, indicators *Streptococcus sanguinis* ATCC 10556, *Lactococcus lactis* strains A5 and C2102, and *Streptococcus anginosus* strains G39 and K11 appeared sensitive to SA-FF22, but not to mutacin K8. Indicators *S. sanguinis* ATCC 10556, *S. anginosus* strains G39, K33 and K11 and *S. dysgalactiae* strain 67 were sensitive to additional (as yet uncharacterized), but apparently *nlmT*-dependent inhibitory substance(s) produced by strain K8.

Fig. 2. (a) C18 reversed-phase HPLC of acidified methanol extract from *S. mutans* strain K8. The flow rate was 1 ml min⁻¹ with an acetonitrile gradient of 0–100% in 55 min. Three 1 ml fractions collected at 25, 26 and 27 min are indicated by the frame and each had inhibitory activity against indicator I1. (b) MALDI-TOF MS analysis of HPLC-purified mutacin K8 (fraction 26). A major peak corresponding to a mass of 2734 Da was observed. (c) Predicted precursor peptides of the four putative lantibiotic structural genes in the *muk* locus (*mukA1*, *mukA2*, *mukA3* and *mukA'*). Putative cleavage sites are indicated by •. Residues predicted to be either dehydrated or involved in lanthionine ring formation are shaded black. Also shown (in grey) is the N-terminal sequence derived from fraction 26 (Fig. 2a) which corresponds to the propeptides MukA1 and MukA3.

Table 4. Deferred antagonism producer (P)-typing of mutacin K8-positive *S. mutans* and their bacteriocin deletion mutants

Degree of inhibition of indicator: —, no inhibition; 1, <1 cm; 2, 1–2 cm; 3, 2–3 cm; 4, >3 cm; (1), resistant colonies within inhibition zone.

<i>S. mutans</i> producer strain*	Test medium	Inhibition of standard indicator strain									P-type
		I1	I2	I3	I4	I5	I6	I7	I8	I9	
K8	BaCa	4	2	3	3	3	4	3	4	(1)	776
K8ΔMutK8	BaCa	—	—	3	—	—	—	—	—	—	100
K8ΔNlmT†	BaCa	4	2	—	2	2	4	3	4	—	676
K8	TsyCa	2	4	4	4	3	3	3	4	3	777
K8ΔMutK8	TsyCa	—	3	4	4	3	3	3	3	2	377
K8ΔNlmT	TsyCa	2	—	—	—	—	2	—	—	—	410

*Producer strain incubation was on BaCa for 22 h at 37 °C in a 5 % CO₂ in air atmosphere.

†The production of mutacin IV (and any other bacteriocins that are secreted via the *nlmT*-encoded transporter) is abrogated in this strain.

Distribution in *S. mutans* of the structural genes for mutacin K8 and other previously characterized mutacins

A set of 26 *S. mutans* strains was screened by PCR to detect the structural genes encoding several known mutacins (Table 6). Strain K8 was PCR-positive for the mutacin IV, mutacin N and mutacin K8 structural genes. The mutacin structural gene most frequently detected, i.e. in 14 (54 %) of the tested strains, was that encoding mutacin IV. PCR-positive reactions for mutacin K8 were given by 9 (35 %) of 26 strains. Strain JH1140 is of particular interest as it is currently being investigated for its anti-caries potential

(Hillman *et al.*, 1998). In the present study strain JH1140 was PCR-positive for mutacin III (also referred to as mutacin 1140), mutacin IV, Smb and mutacin K8.

Comparison of *mukA*-positive *S. mutans* for their sequence and locus size

The sequences of the mutacin K8 structural genes *mukA*1, 2 and 3 have been obtained for nine *S. mutans* strains (JH1140, K18, M46, 4N, K8, D9, K21, G31 and K24). The sequences for all nine strains were identical at the nucleotide level, with the exception of two silent base pair differences in

Table 5. Deferred antagonism inhibitory activity of *S. mutans* K8 and *S. pyogenes* FF22 and their mutated variants against representative differential indicators

Degree of inhibition of indicator: —, no inhibition; 1, <1 cm; 2, 1–2 cm; 3, 2–3 cm; 4, >3 cm.

Differential indicator strain	Degree of inhibition effected by				
	<i>S. pyogenes</i> strain*		<i>S. mutans</i> strain*		
	FF22	EB1	K8	K8ΔMutK8	K8ΔNlmT
<i>S. pyogenes</i> FF22	—	—	2	—	2
<i>S. pyogenes</i> EB1	3	—	3	—	3
<i>S. pyogenes</i> K24 GAS	3	—	2	—	2
<i>S. sanguinis</i> ATCC 10556	2	—	3	3	—
<i>L. lactis</i> A5	3	—	—	—	—
<i>L. lactis</i> C2102	4	—	—	—	—
<i>E. hirae</i> ATCC 9790	4	—	3	—	3
<i>E. hirae</i> lyt14	—	—	—	—	—
<i>S. anginosus</i> G39	4	—	4	4	—
<i>S. anginosus</i> K33	—	—	4	4	—
<i>S. anginosus</i> K11	2	—	3	3	—
<i>S. dysgalactiae</i> 67	—	—	3	3	3
<i>S. equi</i> 1506	2	—	2	—	2

*Producer strain incubation was on BaCa for 22 h at 37 °C in a 5 % CO₂ in air atmosphere.

Table 6. PCR screening of *S. mutans* strains using primers specific for known mutacin structural genes

<i>S. mutans</i> strain	Presence of structural genes of characterized mutacins as determined by PCR						
	Mutacin I	Mutacin II	Mutacin III	Mutacin IV	Mutacin N	Smb	Mutacin K8
H7	–	–	+	+	–	–	–
Ny266	–	–	+	+	–	–	–
54N	–	–	+	+	–	–	–
11b	–	–	+	+	–	–	–
JH1140	–	–	+	+	–	+	+
K49	–	–	+	–	–	–	–
K86	–	–	+	–	–	–	–
31b	–	–	+	–	–	–	–
K47	–	–	+	–	–	–	–
H23	–	–	+	–	–	–	–
K18	–	–	–	+	–	–	+
M46	–	–	–	+	–	–	+
4N	–	–	–	+	–	–	+
K8	–	–	–	+	+	–	+
D9	–	–	–	–	–	–	+
K21	–	–	–	–	–	–	+
G31	–	–	–	–	–	–	+
K24	–	–	–	–	–	–	+
M19	–	+	–	–	–	–	–
UA96	–	+	–	–	–	–	–
N	–	–	–	+	+	–	–
UA159	–	–	–	+	–	–	–
34P	–	–	–	+	–	–	–
UA140	+	–	–	+	–	–	–
GS5	–	–	–	–	–	+	–
K34-1	+	–	–	+	–	+	–

strain K8, neither of which effected any change in the predicted amino acid sequences of the gene products.

The central component of the *muk* locus (i.e. *mukK* to *mukF*) in each of the nine strains was amplified using the primer pair *scnK* and *scnF*. Eight strains yielded amplicons of approximately 10–11 kb. Only *S. mutans* strain 4N differed, its PCR product being approximately 1.5 kb larger. Further sequence analysis of the strain 4N *muk* locus showed that it contained DNA homologous to SMU.1812 of strain UA159, a segment closely resembling a plasmid insertion sequence from *S. mutans* UA855 (GenBank accession no. AF104380). This sequence appears to account for the extra 1.5 kb of sequence within the strain 4N *muk* locus.

Extraction, purification and partial characterization of mutacin K8

Methanol extraction of cells of *S. mutans* strain K8 Δ nlmT (i.e. phenotypically negative for mutacin IV and mutacin N) that had been grown on THB agar yielded putative mutacin K8 inhibitory activity of titre 2 AU ml^{–1} against *M. luteus*. The mutacin K8 activity was bound to a C18 reversed-phase resin and then eluted at approximately 35 % in a 0–100 %

acetonitrile gradient (Fig. 2a). An absorbance peak corresponding to the inhibitor-positive fraction 26 contained a single peptide with a mass of 2734.2 $\pm$ 20 Da when analysed using MALDI-TOF MS (Fig. 2b). N-terminal sequencing of the first six amino acids yielded the sequence Met-Gly-Lys-Gly-Ala-Val. This sequence corresponds exactly to that predicted for the putative propeptides MukA1 and MukA3 (Fig. 2c). Although MukA1 and MukA3 are identical in their predicted propeptide sequences and hence would be expected to co-purify, they differ by three amino acids in their leader peptide sequences. The relative expression levels in strain K8 of the MukA1 and MukA3 propeptides have not been determined.

Inhibitory spectra of mutacin K8 and SA-FF22

Some differences were noted in the relative strain targeting activities of purified preparations of mutacin K8 and SA-FF22. For example, strains *L. lactis* C2102 and *S. anginosus* G39 appeared sensitive to SA-FF22, but not to mutacin K8. Furthermore, although strain EB1 (the SA-FF22-negative derivative of strain FF22) was more than 32-fold more sensitive than the parent strain FF22 to SA-FF22, it appeared to be only marginally (i.e. twofold) more sensitive than

strain FF22 to mutacin K8. By contrast, strain K8 Δ mutK8 (the mutacin K8-negative derivative of strain K8) did not seem to differ from the parent strain K8 in its susceptibility to the available test concentrations of either mutacin K8 or SA-FF22.

DISCUSSION

Deferred antagonism tests of a group of *S. mutans* strains indicated that three produced inhibitory activity similar to that of *S. pyogenes* strain FF22 (the prototype producer of the lantibiotic SA-FF22). The main basis for this perceived similarity was that all three of these *S. mutans* strains had relatively less inhibitory activity against strain FF22 than against its SA-FF22-negative derivative (strain EB1). This was taken to indicate specific lantibiotic cross-immunity of strain FF22 to the inhibitory products of these *S. mutans* strains. Similarly to the findings with SA-FF22 (Jack & Tagg, 1992), inhibitor production by these three *S. mutans* strains appeared to be completely abrogated when either 1 % MgCl₂ or 2 mg trypan blue ml⁻¹ was added to the test medium (results not shown).

Although five ORFs having close homology to the SA-FF22 regulatory and immunity genes are present in the *S. mutans* strain UA159 genome, no genes having homology to those required for SA-FF22 biosynthesis are detectable, indicating that the locus is inactive. Similarly, although more than 95 % of *S. pyogenes* harbour the structural gene *salA1* for the lantibiotic salivaricin A1, it seems to date that only serotype M4 strains contain the entire *salA1* locus and thus are capable of producing biologically active salivaricin A1 (Upton *et al.*, 2001; Wescombe *et al.*, 2006).

In the present study, primers for the 2.5 kb region between SMU.1815 (*scnR*-like) and SMU.1811 (*scnF*-like) in the genome of *S. mutans* strain UA159 yielded an approximately 10 kb amplification product from strain K8. The additional DNA in strain K8 was sequenced and four ORFs (*mukA1*, *mukA2*, *mukA3* and *mukA'*) encoding putative type AII lantibiotic prepeptides were identified in addition to ORFs having homology to typical lantibiotic modification (*mukM*) and transport (*mukT*) genes. The entire mutacin K8 locus resembles that of the SA-FF22 locus in *S. pyogenes* strain FF22 (McLaughlin *et al.*, 1999), indicating that the two loci probably share a common ancestry. Interestingly, however, *mukK* and *mukR* are in the same transcriptional orientation as the putative mutacin K8 immunity and biosynthetic genes, whereas *scnR* and *scnK* are encoded by the complementary strand within the SA-FF22 locus.

The presence in strain K8 of three almost identical copies of a lantibiotic structural gene has a precedent in the ruminococcin A locus of *Ruminococcus gnavus* (Gomez *et al.*, 2002). Interestingly, all three RumA structural genes are transcribed and may also be translated (Gomez *et al.*, 2002). In the case of mutacin K8, the putative *mukA2* translation product would have a significantly different structure, being

capable of forming just two of the three lanthionine rings predicted to be present in the MukA1 and MukA3 propeptides. It seems that the structural differences imposed upon MukA2 by the absence of one ring structure will necessarily result in some alternative function(s) for that peptide. One possibility is that mutacin K8 may be a two-component lantibiotic, consisting of MukA1 and/or MukA3 in combination with MukA2 or MukA'. However, fractionation of a mutacin K8 preparation identified only a single peak having inhibitory activity, and this fraction contained just one peptide, the sequence of which matched the predicted translation product of *mukA1* and/or *mukA3*. However, it is possible that the MukA2 and MukA' propeptides have no activity on their own, but that they enhance the activity of the MukA1/A3 peptides. Interestingly, the MukA1/MukA3 peptide preparation had only slightly more inhibitory activity against strain EB1 than against strain FF22, a finding consistent with the relatively minor apparent susceptibility differences exhibited by strains EB1 and FF22 when used as indicators in deferred antagonism tests of strain K8 Δ NlmT.

Although no function has yet been defined for the putative product of *scnA'* within the SA-FF22 locus, it is however known that SA-FF22 is not the specific signal peptide for its own upregulation (Wescombe, 2002). Consequently it can be speculated that the *scnA'* translation product may fulfil this function. The significant homology of *mukA'* and *scnA'* supports a possible similar function for the peptide product of *mukA'*.

Inactivation of the *muk* locus demonstrated that mutacin K8 accounts for the majority of observed antimicrobial activity of strain K8 in deferred antagonism tests on BaCa against the nine standard indicators. The exception is the activity against indicator I3 (*S. anginosus*). This latter activity could, however, be eliminated by inactivation of *nlmT*, which encodes an ABC transporter known to export non-lantibiotic bacteriocins such as mutacin IV (Hale *et al.*, 2005a). Further inhibitory spectrum testing of this *nlmT*-knockout strain (predicted to produce only mutacin K8 on BaCa) indicated that *M. luteus* as well as all tested *S. pyogenes* and some *L. lactis*, *Streptococcus constellatus* and *S. uberis* strains were susceptible. Interestingly, the lantibiotic-producing *L. lactis* strains A5 (a producer of nisin Z) and C2102 (a producer of lacticin 481) appeared relatively less sensitive than the non-lantibiotic-producing *L. lactis* strain I6 to the mutacin K8-producer strain K8 Δ NlmT. On the other hand both A5 and C2102 appeared quite sensitive to SA-FF22, making them useful indicators for differentiating between mutacin K8 and SA-FF22. This apparent susceptibility difference was confirmed by comparing the activity spectra of purified preparations of SA-FF22 and mutacin K8. In contrast, mutacin K8 (but not SA-FF22) is inhibitory to *S. dysgalactiae* strain 67 (which harbours the SA-FF22 structural gene). These findings indicate that the SA-FF22 immunity gene products do not provide very effective cross-protection against mutacin K8.

This study clearly indicates that the demonstration of a particular mutacin structural gene does not necessarily facilitate prediction of the host strain's anti-bacterial spectrum, since many *S. mutans* encode a wide variety of mutacins. The most common bacteriocin structural gene pairing for strains having the *muk* locus was with the mutacin IV (*nlmA* and *nlmB*) locus, with 5 of the 9 *muk*-positive strains having both loci. The *muk* locus appears to be relatively widespread within *S. mutans*, being detected in 9 (35 %) of 26 tested strains. Indeed, on the basis of our study its occurrence in *S. mutans* appears similar to that of mutacin III, as determined in both the present (38 %) and other (25 %) studies (Bekal-Si Ali *et al.*, 2002). Interestingly, however, we found only one strain (JH1140) that contained the structural genes for both mutacin III and mutacin K8. We speculate that, on the basis of its high prevalence, mutacin K8 probably provides the *S. mutans* producer strain with a significant ecological advantage within the high-density, heterogeneous Gram-positive bacterial populations present in supra-gingival dental plaque. This benefit could result either from the direct inhibition of competitors or alternatively by providing a measure of protective immunity against various of the closely similar bacteriocins produced by members of the mutans or other streptococcal species that abound within the indigenous oral consortium of humans.

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