Frequency and expression of mutacin biosynthesis genes in isolates of *Streptococcus mutans* with different mutacin-producing phenotypes

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The aim of this study was to analyse the frequency and expression of biosynthesis genes in 47 *Streptococcus mutans* isolates with different mutacin-producing phenotypes. Detection of the frequency and expression of genes encoding mutacin types I, II, III and IV were carried out by PCR and semi-quantitative RT-PCR, respectively, using primers specific for each type of biosynthesis gene. In addition, a further eight genes encoding putative bacteriocins, designated *bsm* 283, *bsm* 299, *bsm* 423, *bsm* 1889c, *bsm* 1892c, *bsm* 1896, *bsm* 1906c and *bsm* 1914, were also screened. There was a high phenotypic diversity; some *Streptococcus mutans* isolates presented broad antimicrobial spectra against other *Streptococcus mutans* clinical isolates, including bacteria resistant to common antibiotics, as well as *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Enterococcus faecalis* and *Streptococcus pyogenes*. The expression frequency of the *bsm* gene was higher than that of the previously characterized mutacins (I–IV). There was no positive correlation between the number of indicator strains inhibited (antimicrobial spectra) and the number of biosynthesis genes expressed (Spearman correlation test, $r = -0.03$, $P > 0.05$). In conclusion, the high diversity of mutacin-producing phenotypes, associated with high frequency of expression of the biosynthesis genes screened, reveals a broad repertoire of genetic determinants encoding antimicrobial peptides that can act in different combinations.

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INTRODUCTION

Streptococcus mutans is considered a major aetiological agent of human dental caries due to its capacity for adherence, acid resistance, acidogenicity, resistance to other stress conditions and production of mutacins (Bowden & Hamilton, 1998; Napimoga *et al.*, 2005). In addition, *Streptococcus mutans* produces mutacins or antimicrobial peptides that could confer ecological advantage to cariogenic species in diverse bacterial communities such as saliva and dental biofilms (Parrot *et al.*, 1990; Balakrishnan *et al.*, 2002).

In a previous study, we showed that the majority of *Streptococcus mutans* strains produce mutacin-like substances (Kamiya *et al.*, 2005a); of 319 isolates of *Streptococcus mutans* isolated from caries-active and caries-free individuals, more than 70% were able to produce one or more bacteriocin-like substances *in vitro*. However, the low frequency of biosynthesis genes for mutacins previously characterized in these isolates suggests a high diversity of bacteriocin genetic determinants in *Streptococcus mutans* (Kamiya *et al.*, 2005b). Despite this

great diversity, little has been revealed about the biochemical structures of mutacins. In addition, many mutacins have not yet been identified.

Most *Streptococcus mutans* bacteriocins characterized to date belong to the class I lantibiotics (mutacins I, II, III, 1140, BNy-266, and SmbA and SmbB) (Hillman *et al.*, 1998; Qi *et al.*, 1999a, b, 2000, 2001; Yonezawa & Kuramitsu, 2005). Class I bacteriocins comprise two subgroups according to their primary peptide sequences (de Vos *et al.*, 1995; Sahl & Bierbaum, 1998). Subgroup AI contains the nisin-like lantibiotics, as well as subtilin, epidermin, pep5, and mutacins I and III as the most thoroughly characterized members. Subgroup AII consists of lantibiotics including lactacin 481, SA-FF22, salivaricin, variacin and mutacin II (de Vos *et al.*, 1995; Sahl & Bierbaum, 1998; Qi *et al.*, 2000).

Class II bacteriocins include non-lantibiotics, divided into subclass IIa, which contains the pediocin-like substances, and subclass IIb, which is composed of two synergic peptides (Nes & Holo, 2000). According to Ajdic *et al.* (2002), the genome sequence of reference strain *Streptococcus mutans* UA159 does not possess any genetic determinants that encode lantibiotic mutacins. However,

Abbreviation: CSP, competence-stimulating peptide.

other studies have shown that *Streptococcus mutans* UA159 produces the non-lantibiotic mutacin IV, a non-lantibiotic class IIb bacteriocin encoded by the *nImA* and *nImB* genes (Qi *et al.*, 2001), as well as an additional, as-yet-unidentified, inhibitory agent (Hale *et al.*, 2005b).

Furthermore, analysis of the *Streptococcus mutans* genome sequence (Ajdic *et al.*, 2002) has revealed ten small open reading frames with high similarity to the leader peptides of NImA and NImB that encode class IIb bacteriocins (non-lantibiotics), each possessing a double-glycine-type leader sequence similar to that of NImA and NImB (Fig. 1) (Hale *et al.*, 2005a; van der Ploeg, 2005). The putative bacteriocins, designated Bsm (bacteriocin *Streptococcus mutans*), range in size from 47 to 87 aa, have leader peptides of 22–25 aa and contain a double glycine motif that can be recognized by the ComAB processing and export system (NImTE). Some of the genes encoding the putative bacteriocins have been found located in tandem, indicating that they might act cooperatively, as is typical for class IIb bacteriocins; these peptides may represent a large repertoire of antimicrobial substances produced by *Streptococcus mutans* (Hale *et al.*, 2005a; van der Ploeg, 2005).

In the present study, the frequency and expression of characterized mutacin biosynthesis genes was determined, as well as lantibiotics represented by mutacins I, II and III and non-lantibiotic mutacin IV. Additionally, we demonstrated the frequency and expression of the uncharacterized mutacins Bsm 283, Bsm 299, Bsm 423, Bsm 1889c, Bsm 1892c, Bsm 1896, Bsm 1906c and Bsm 1914 in different mutacin-producing *Streptococcus mutans* phenotypes. The inhibitory spectra were obtained against microbial species of odontological and medical interest.

METHODS

Strains and *Streptococcus mutans* isolates. We selected 47 different genotypes of *Streptococcus mutans* (Sm 1 to Sm 47) for this study; genotypes were isolated from caries-active individuals (Kamiya *et al.*, 2005a), and from caries-free mother–child pairs (Flório *et al.*, 2004; Klein *et al.*, 2004), identified in previous studies.

NImA	MDTQAFEQFDVMSQTLSTVEGG	
Bsm_283	MDTMAFENFDEIDMNHLSIEGG	FDVKG
Bsm_299	MNTKMEQFETMDAETLSHVTGG	GLYDG
Bsm_423	MNTQAFEQFNVDNEALSTVEGG	GMIRC
Bsm_1889c	MMNTRTLEQFDAMDVDMLAAVEGG	NWGQC
Bsm_1892c	MKTQTEIWKRFEALDTADLAIQGG	SENIA
Bsm_1896	MMEIKALDQFETMDTDLAAVEGG	FGWDS
Bsm_1906c	MNTHVLEQFDVMSQVPSAIEGG	GCSWK
Bsm_1914	MNTQAFEQFNVDNEALSAVEGG	GRGWN
NImB	MELNVNYYKSLTNDELSEVFEGG	SGSLN

Fig. 1. CLUSTAL W sequence alignment of NImA and NImB (the pre-peptides of the two-component non-lantibiotic mutacin IV) with other pre-peptides (in *Streptococcus mutans* UA159) containing the double-glycine motif. Adapted from Hale *et al.* (2005b) and van der Ploeg (2005).

For mutacin activity testing and phenotyping, the following 16 *Streptococcus* spp. were used as indicator strains: *Streptococcus mutans* UA159, *Streptococcus mutans* UA130, *Streptococcus mutans* CCT 3440, *Streptococcus mutans* 32K, *Streptococcus sobrinus* ATCC 27607, *Streptococcus sobrinus* 6715, *Streptococcus mitis* A, *Streptococcus mitis* ATCC 903, *Streptococcus salivarius* ATCC 25975, *Streptococcus salivarius* 66.4, *Streptococcus sanguinis* CR311, *Streptococcus sanguinis* M5, *Streptococcus sanguinis* ATCC 10556, *Streptococcus sanguinis* ATCC 15300, *Streptococcus oralis* PB182 and *Streptococcus criceti* HS6. In addition, 14 *Streptococcus mutans* isolates were used in the test as indicator strains.

Sixteen high inhibitory spectra mutacin-producing isolates were selected and submitted to an antagonism method against nine bacterial and five fungal species, comprising: *Enterococcus faecalis* ATCC 10100, *Enterococcus faecalis* ATCC 14506, *Enterococcus faecalis* ATCC 19433^T, *Enterococcus faecalis* ATCC 29212, *Staphylococcus aureus* ATCC 6538DR, *Staphylococcus aureus* ATCC 12692, *Staphylococcus aureus* ATCC 19095, *Staphylococcus aureus* ATCC 27664, *Staphylococcus epidermidis* ATCC 12228, *Streptococcus pyogenes* ATCC 8668 (A group), *Streptococcus pyogenes* ATCC 12344^T, *Streptococcus pyogenes* ATCC 19615, *Streptococcus gordonii* Chalis, *Lactobacillus casei* L324M, *Lactobacillus acidophilus* A, *Pseudomonas aeruginosa* ATCC 25619, *Escherichia coli* NCTC 8196, *Candida albicans* ATCC 36801, *C. albicans* ATCC 36802, *C. albicans* CBS 562, *Candida dubliniensis* CBS 7987, *Candida krusei* CBS 573, *Candida parapsilosis* CBS 604 and *Candida tropicalis* CBS 94. Additionally, mutacin-producing *Streptococcus mutans* ($n=16$) were also used as indicator strains.

Antagonism method. The genotypes of *Streptococcus mutans* were characterized as producers of mutacin-like substances by the antagonism method, as described previously by our group with some modifications (Kamiya *et al.*, 2005a). Briefly, frozen *Streptococcus mutans* cultures were reactivated in 5 ml brain heart infusion broth (Difco) and incubated at 37 °C with 10 % CO₂ for 12 h. The strain cultures ($\sim 10^8$ c.f.u. ml⁻¹, measured at OD₅₅₀) were inoculated equidistantly in 1.5 % (w/v) trypticase soy agar (TSA; Difco) using a 0.6 mm needle. After a 48 h incubation at 37 °C and 10 % CO₂, the plates were overlaid with 4.5 ml soft 0.8 % (w/v) TSA containing 0.5 ml of an overnight trypticase soy broth (TSB; Difco) culture ($\sim 10^8$ c.f.u. ml⁻¹) of the indicator strain. After overnight incubation at 37 °C, the diameter of the inhibition zones was measured. The isolates were recorded as mutacin active against the indicator strain if the diameter was 6 mm or greater (Wu *et al.*, 2004). The isolates were tested in duplicate for mutacin activity.

Mutacin activity profiles. Thirty *Streptococcus* spp. indicator strains were used. For every three indicator strains, a score of between 0 and 7 was given to the isolates depending on which of the isolates were sensitive to the mutacin produced (Table 1). In this way, a ten-figure profile characterized every isolate. These profiles were regarded as distinct when one or more of the figures were different (van Loveren *et al.*, 2000).

Kirk–Bauer disc diffusion method. Bacterial indicator strains of medical interest, as well as *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Enterococcus faecalis* and *Streptococcus pyogenes*, were submitted to the Kirk–Bauer disc diffusion method, which was performed in accordance with Clinical and Laboratory Standards Institute standards (CLSI, 2006). The antimicrobials used were: benzylpenicillin, ampicillin, amoxicillin, imipenem, vancomycin, azithromycin, streptomycin and tetracycline. The bacterial strains *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were used as quality controls for the discs (Laborclin).

PCR screening of mutacin genes. Total DNA was extracted using a Master Pure DNA Purification kit (Epicentre Biotechnologies),

Table 1. System used to transform the bacteriocin production against three indicator strains into a unique numerical classification (van Loveren *et al.*, 2000)

+, Bacteriocin production against indicator strains; –, no bacteriocin production against indicator strains.

Score	Indicator strain		
	1	2	3
0	–	–	–
1	+	–	–
2	–	+	–
3	+	+	–
4	–	–	+
5	+	–	+
6	–	+	+
7	+	+	+

according to the manufacturer's instructions. The mutacin-producing strains were grown in 3 ml brain heart infusion broth (Oxoid) for 18 h at 37 °C and 10% CO₂. Aliquots of 1.5 ml culture were submitted to centrifugation (13 000 g at 4 °C for 5 min) and DNA was extracted from the pellet. The DNA purity was determined by calculating the A_{260}/A_{280} ratio.

The detection of biosynthesis genes encoding mutacin types I, II, III and IV (Qi *et al.*, 1999a, b, 2001) and different *bsm* genes was performed by PCR using primers specific to each type of gene. Primers for the genes encoding mutacins or bacteriocins were designed based on sequences obtained from GenBank (<http://www.ncbi.nlm.nih.gov>) (Table 2) using the Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi).

PCR amplification was performed using a GeneAmp PCR System 2400 (Perkin Elmer). The 50 µl reactions consisted of 1× PCR buffer containing 2.5 mM MgCl₂, 200 µM each dNTP, 0.3 µM each oligonucleotide primer, 1.25 U *Taq* DNA polymerase (Life Technologies) and 50 ng template DNA. In addition to the strains being tested, purified genomic DNA from *Streptococcus mutans* UA159 was used as a positive control for mutacin gene type IV and *bsm* genes. Distilled water was used as a negative control in each PCR.

The PCR conditions were optimized using, as controls, DNA from *Streptococcus mutans* UA159 and the clinical isolate carrier *mutAI*, *mutAII* and *mutAIII* genes (detected in a previous study; Kamiya *et al.*, 2005b). The PCR conditions comprised initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 50 °C for 1 min and extension at 72 °C for 2 min, with a final extension at 72 °C for 7 min. The PCR products were analysed by electrophoresis in a 1.0% agarose gel stained with 0.3 mg ethidium bromide ml⁻¹. A 100 bp DNA ladder was included in each gel.

RNA extraction, synthesis of cDNA and semi-quantitative RT-PCR. Mutacin-producing strains that were shown to have the screened structural genes by PCR were submitted to RNA extraction

Table 2. PCR and RT-PCR primers of mutacins I–IV and the *bsm* genes

Protein	Primer	Primer sequences	GenBank accession no.	Amplicon (bp)
Mutacin I (MutAI)	mutAI_F	TAGAAGTCCTTGGTACTGAA	AF238860	163
	mutAI_R	TTGAAACTAGGATTTTTCAC		
Mutacin II (MutAII)	mutAII_F	CAGTAACGCAGTAGTTCCTT	U40620	162
	mutAII_R	TTAACAGCAAGTGAAAACAT		
Mutacin III (MutAIII)	mutAIII_F	CAATTATTAGAAGTCCTTGG	AF154675	194
	mutAIII_R	TATTGAACTACCTGTCCTT		
Mutacin IV (NlmA)	nImA_F	CAATTTGTATGAATGGACAG	NC_004350*	154
	nImA_R	CTACACAATATGGGGTAACA		
Mutacin IV (NlmB)	nImB_F	AGTTTTTGGTGGAGATAAAC	NC_004350*	156
	nImB_R	GGAAAACTACAGATCCAA		
Bsm_283	Bsm_283F	GCTAGTATTGAGGGAGGAT	NC_004350*	153
	Bsm_283R	ATAAATAACTGCAGCACCT		
Bsm_299	Bsm_299F	AAACTATGGATGCTGAAAC	NC_004350*	160
	Bsm_299R	GAAGCCCAGCTATTTACTA		
Bsm_423	Bsm_423F	GTAATGGATAATGAAGCAC	NC_004350*	154
	Bsm_423R	CAGATACTGTACCAACGAC		
Bsm_1889c	Bsm_1889cF	GCTCCTGATATAGCTCCTA	NC_004350*	154
	Bsm_1889cR	AATGTATTGTAGGGACAGG		
Bsm_1892c	Bsm_1892cF	CGAGCATAGCTTACAAC	NC_004350*	130
	Bsm_1892cR	CACAACTGAAATCTGGA		
Bsm_1896	Bsm_1896F	GTATCTGGAGAGGTTTCAA	NC_004350*	148
	Bsm_1896R	ACACCAGCTCCTGATATAC		
Bsm_1906	Bsm_1906F	CTATTCCAAGGACTCCAC	NC_004350*	140
	Bsm_1906R	ACGTCATGGATAGTCAAGT		
Bsm_1914c	Bsm_1914cF	AATAGCATATGGACCAAGA	NC_004350*	141
	Bsm_1914cR	ACGTAATGGATAATGAAGC		

*Based on the *Streptococcus mutans* UA159 genome.

Table 3. Antimicrobial activities of the mutacin-producing phenotypes ($n=47$) against 30 *Streptococcus* sp. indicator strains

Spectrum	Producer strain	Number of inhibited strains of different bacterial species								Number (%) of inhibited indicator strains
		<i>S. mutans</i> ($n=47$)	<i>S. mutans</i> ($n=4$)	<i>S. sobrinus</i> ($n=2$)	<i>S. mitis</i> ($n=2$)	<i>S. salivarius</i> ($n=2$)	<i>S. sanguinis</i> ($n=4$)	<i>S. oralis</i> ($n=1$)	<i>S. criceti</i> ($n=1$)	<i>S. mutans</i> isolates ($n=14$)
Broad spectrum	Sm 1	4	2	2	2	4	1	1	12	28 (93.3)
	Sm 2	4	2	2	2	4	1	1	12	28 (93.3)
	Sm 3	4	2	2	1	4	1	1	12	27 (90.0)
	Sm 4	4	1	2	2	4	1	1	12	27 (90.0)
	Sm 5	4	1	2	1	4	1	1	12	26 (86.6)
	Sm 6	2	1	2	2	3	1	1	13	25 (83.3)
	Sm 7	4	1	2	2	2	1	1	12	25 (83.3)
	Sm 8	4	1	2	1	4	1	1	9	25 (83.3)
	Sm 9	4	1	2	1	4	1	1	9	25 (83.3)
	Sm 10	4	2	2	2	4	1	1	9	25 (83.3)
	Sm 11	4	1	2	1	3	1	0	11	23 (76.6)
	Sm 12	3	1	2	1	4	1	1	8	23 (76.6)
	Sm 13	4	1	2	1	4	1	1	9	23 (76.6)
	Sm 14	3	1	2	1	4	1	1	8	21 (70.0)
	Sm 15	0	1	2	2	3	1	0	5	14 (46.7)
	Sm 16	1	0	0	0	0	0	0	12	13 (43.3)
Low spectrum	Sm 17	0	2	2	2	3	1	1	0	11 (36.6)
	Sm 18	0	2	2	2	3	1	1	0	11 (36.6)
	Sm 19	0	2	2	1	3	1	1	0	10 (33.3)
	Sm 20	0	2	2	1	4	1	1	0	10 (33.3)
	Sm 21	0	1	2	2	2	1	1	0	9 (30.0)
	Sm 22	2	1	1	2	0	0	0	2	8 (26.6)
	Sm 23	0	0	1	0	1	0	1	2	5 (16.6)
	Sm 24	0	0	0	0	2	1	0	0	3 (10.0)
	Sm 25	0	0	0	0	2	1	0	0	3 (10.0)
	Sm 26	0	0	1	0	2	0	0	0	3 (10.0)
	Sm 27	0	0	1	0	2	0	0	0	3 (10.0)
	Sm 28	0	0	0	0	2	1	0	0	3 (10.0)
	Sm 29	0	0	0	0	2	1	0	0	3 (10.0)
	Sm 30	0	0	0	0	2	1	0	0	3 (10.0)
	Sm 31	0	0	1	0	0	1	0	0	2 (6.6)
	Sm 32	0	0	1	0	0	1	0	0	2 (6.6)
	Sm 33	0	0	0	0	1	0	0	0	1 (3.3)
	Sm 34	0	0	0	0	1	0	0	0	1 (3.3)
	Sm 35	0	0	0	0	1	0	0	0	1 (3.3)
	Sm 36	0	0	0	0	1	0	0	0	1 (3.3)
	Sm 37	0	0	0	0	1	0	0	0	1 (3.3)
	Sm 38	0	0	0	0	1	0	0	0	1 (3.3)
	Sm 39	0	0	0	0	1	0	0	0	1 (3.3)
Non-producer	Sm 40	0	0	0	0	0	0	0	0	0 (0)
	Sm 41	0	0	0	0	0	0	0	0	0 (0)
	Sm 42	0	0	0	0	0	0	0	0	0 (0)
	Sm 43	0	0	0	0	0	0	0	0	0 (0)
	Sm 44	0	0	0	0	0	0	0	0	0 (0)
	Sm 45	0	0	0	0	0	0	0	0	0 (0)
	Sm 46	0	0	0	0	0	0	0	0	0 (0)
	Sm 47	0	0	0	0	0	0	0	0	0 (0)

and a semi-quantitative RT-PCR technique to verify the possible expression of these genes. The strains were grown in 40 ml TSB at 37 °C, 10 % CO₂, up to the final exponential phase at 10–12 h. The planktonic cells were obtained by centrifugation and submitted to RNA extraction using a phenol/chloroform method (Qi *et al.*, 1999a).

Residual DNA in the extracted RNA was removed with DNase I (Gibco), according to the manufacturer's instructions. cDNA synthesis was performed with 24 ng total RNA using a random primer mix of Ea1, Ea7, Es1, Es3 and Es8 (Chia *et al.*, 2001) and the enzyme SuperScript III reverse transcriptase (Gibco), following the

Table 4. Antimicrobial activity of the broad-spectrum mutacin-producing phenotypes ($n=16$) against 36 indicator strains

Producer strain ($n=16$)	Number of inhibited strains of different bacterial and fungi species							Total no. (%) of inhibited indicator strains
	<i>Lactobacillus</i> spp. ($n=2$)	<i>Staphylococcus aureus</i> ($n=4$)	<i>Staphylococcus epidermidis</i> ($n=1$)	<i>Streptococcus pyogenes</i> ($n=3$)	<i>Enterococcus faecalis</i> ($n=3$)	<i>Streptococcus mutans</i> producer strains ($n=16$)	<i>Candida</i> spp. ($n=7$)	
Sm 1	2	4	1	3	0	14	5	29 (80.5)
Sm 2	2	4	1	3	0	14	5	29 (80.5)
Sm 3	1	2	0	3	2	1	0	9 (25.0)
Sm 4	2	4	1	3	1	7	5	23 (63.8)
Sm 5	2	4	1	3	0	15	3	28 (77.7)
Sm 6	1	4	1	3	2	15	2	28 (77.7)
Sm 7	1	1	0	3	0	2	0	7 (19.4)
Sm 8	2	4	1	3	1	12	5	28 (77.7)
Sm 9	2	4	1	3	0	11	2	23 (63.8)
Sm 10	2	4	1	3	1	1	3	15 (41.6)
Sm 11	2	4	1	3	0	15	5	30 (83.3)
Sm 12	2	4	1	3	0	15	4	29 (80.5)
Sm 13	1	1	0	3	2	0	0	7 (19.4)
Sm 14	2	4	1	3	0	14	4	28 (77.7)
Sm 15	1	0	0	2	0	0	0	3 (8.3)
Sm 16	0	0	0	3	0	7	0	10 (27.7)

manufacturer's protocol, with some modifications. The reaction mix contained the total RNA, 20 μ M random primer mix, 10 mM dNTPs and DEPC-treated H_2O to 10 μ l. The solution was heated at 65 °C for 5 min and cooled at 4 °C for 1 min. The following were then added to the reaction mixture (final concentrations): 1 $\times$ RT buffer, 25 mM $MgCl_2$, 0.1 M DTT, 40 U RNase OUT and 200 U SuperScript III reverse transcriptase, and the total volume of 20 μ l containing the cDNA was submitted to the following thermal cycle: 25 °C for 10 min, 50 °C for 50 min, 85 °C for 5 min and 4 °C for 1 min. For each RNA sample, the cDNA synthesis reaction was also carried out without reverse transcriptase to identify contamination by residual genomic DNA. For maximum efficiency, the RT-PCR primers were designed to generate amplicons ranging from 130 to 200 bp (Table 2).

A 1 μ l volume of cDNA was used as template in the PCR (total volume 25 μ l) containing 50 mM $MgCl_2$, 0.3 mM each specific primer (Table 2), 200 μ M dNTPs, 1.25 U *Taq* DNA polymerase (Gibco) and 1 $\times$ PCR buffer. All samples of cDNA were tested with primers for the 16S rRNA gene, a constitutively transcribed control gene whose expression is invariant under the experimental conditions used. The primer set 16S/forward (5'-CGGCAAGCTAATCTCTGAAA-3') and 16S/reverse (5'-GCCCCCTAAAAGGTTACCTCA-3') was designed based on the *Streptococcus mutans* UA159 genome (GenBank accession no. NC_004350). Controls for the RT-PCR included reaction mixtures without template cDNA to rule out the presence of contaminating DNA and/or the formation of primer dimers.

The amplicons were visualized on a 2% agarose gel stained with ethidium bromide (0.3 mg ml⁻¹). The positive controls and a 100 bp DNA ladder were included in each gel.

RESULTS AND DISCUSSION

In the present study, about 83% (39/47) of the *Streptococcus mutans* strains analysed showed mutacin activity against one or more of the indicator strains (Table 3). Mutacin production frequency in *Streptococcus*

mutans can vary from 70 to 100% depending on the conditions of the tests and the indicator strains used (Rogers *et al.*, 1979). The inhibition zone sizes for producer strains varied from 6 to 30 mm in diameter. On average, 34.1% (16/47) of these isolates presented a broad spectrum, inhibiting more than 43% of the indicator strains of *Streptococcus* spp., including *Streptococcus mutans* isolates, and 48.9% (23/47) presented a low spectrum, inhibiting from 3 to 36% of the indicator strains of *Streptococcus* spp. (Table 3).

Some mutacin-like substances presented broad antimicrobial spectra. These inhibitory substances were able to kill or inhibit the growth of indicator strains of medical interest that are resistant to some commonly used antibiotics, as well as *Enterococcus faecalis*, *Streptococcus pyogenes*, *Staphylococcus aureus* and *Staphylococcus epidermidis* (Tables 4 and 5). These results emphasize the importance of mutacins in future pharmacology applications, although more studies are necessary to determine their use as a safe antimicrobial agent. There was no antimicrobial activity against *Streptococcus gordonii* and Gram-negative bacteria, as well as *P. aeruginosa* and *Escherichia coli*.

As shown in Fig. 2, of the characterized mutacins, the most frequent biosynthesis gene among the isolates was *mutAI* (29.1%), although the *nlmA* and *nlmB* genes were more highly expressed in mutacin IV (18.7%). Five broad-spectrum isolates and one low-spectrum strain produced mutacin type III. The low frequency of genetic determinants of the characterized mutacins detected in this study is consistent with previous studies (Longo *et al.*, 2003; Kamiya *et al.*, 2005b). On the other hand, some phenotypes presented amplicons and positive gene expression for

Table 5. Susceptibility profiles of the indicator strains of medical interest to some commonly used antibiotics

Results were obtained using the Kirk–Bauer disc diffusion method. Nomenclature based on CLSI recommendations: S, sensitive; MS, moderately sensitive; I, intermediate; R, resistant.

Indicator strain of medical interest (n=14)	Common antibiotics								Mutacin activities	
	Penicillin (10 U)	Ampicillin (10 µg)	Amoxicillin (10 µg)	Imipinem (10 µg)	Vancomycin (30 µg)	Azithromycin (15 µg)	Streptomycin (10 µg)	Tetracycline (30 µg)	No. (%) of mutacin-producing strains with inhibitory activity*	Mean ± SD of inhibition zone (mm)
<i>Enterococcus faecalis</i> ATCC 10100	R	MS	R	I	I	R	R	I	6 (37.5)	(6.3 ± 0.8)
<i>Enterococcus faecalis</i> ATCC 14506	R	R	R	S	R	R	R	R	0	–
<i>Enterococcus faecalis</i> ATCC 19433 ^T	R	R	R	S	R	R	R	R	0	–
<i>Enterococcus faecalis</i> ATCC 29212	R	R	R	S	R	R	R	R	3 (18.7)	(6.0 ± 0.0)
<i>Streptococcus pyogenes</i> ATCC 8668	R	S	S	S	S	R	R	S	15 (93.7)	(11.0 ± 2.7)
<i>Streptococcus pyogenes</i> ATCC 12344 ^T	R	R	R	S	S	R	R	R	16 (100)	(20.0 ± 0.0)
<i>Streptococcus pyogenes</i> ATCC 19615	S	S	S	S	R	R	R	I	16 (100)	(20.0 ± 0.0)
<i>Staphylococcus aureus</i> ATCC 19095	S	S	S	S	I	R	R	R	11 (68.7)	(13.2 ± 1.8)
<i>Staphylococcus aureus</i> ATCC 27664	S	S	S	S	I	R	R	R	12 (75.0)	(12.0 ± 2.4)
<i>Staphylococcus epidermidis</i> ATCC 12228	R	R	R	S	S	R	R	R	11 (68.7)	(13.6 ± 0.8)
<i>P. aeruginosa</i> ATCC 25619	†	I	R	S	R	R	R	R	0	–
<i>E. coli</i> NCTC 8196	†	R	R	S	R	R	S	S	0	–

*Inhibitory activity detected by the antagonism method (inhibition diameters varied between 6 and 20 mm).

†No nomenclature.

Fig. 2. Frequency (□) and expression (■) of genes encoding characterized and uncharacterized mutacins (*bsm* genes) in *Streptococcus mutans* isolates.

mutacins I, II, III and IV, but did not reveal the same antimicrobial spectra. One possible explanation is that the modification of only one amino acid has been shown previously to alter or eliminate the activity of certain bacteriocins (Rollema *et al.*, 1995; Chan *et al.*, 1996). Furthermore, possible polymorphisms of biosynthesis genes or in the cluster of genes related to mutacin production may account for differences in virulence among *Streptococcus mutans* strains. In addition, the inhibition zone could also result from the production of more than one inhibitory substance.

Mutacin IV was produced by 17% of isolates ($n=8$), although it presented low antimicrobial spectra with activity mainly against initial colonizers, as well as *Streptococcus sanguinis* and *Streptococcus oralis*. In contrast, Hale *et al.* (2005a) demonstrated the broad antimicrobial spectra of mutacin IV produced by *Streptococcus mutans* UA159 against a panel of 84 mutacin-sensitive indicator bacteria consisting of 74 streptococcal strains, eight strains of *Lactococcus lactis* and two strains of *Micrococcus luteus*. The differences between the method and indicator strains used hinder a comparison of the results.

Mutacin IV is encoded by the *nlmA* and *nlmB* genes, which are probably organized in an operon (Qi *et al.*, 2001). Genetic dissection of the *nlmAB* locus does not support the hypothesis that mutacin IV is a two-component mutacin and thus the role, if any, of NlmB remains enigmatic (Hale *et al.*, 2005a). In our study, two strains showed expression of *nlmA* or *nlmB* and distinct phenotypic profiles (Table 6). The individual roles of NlmA and NlmB in mutacin IV activity could not be identified in the present study.

Recently, analysis of the *Streptococcus mutans* genome sequence revealed ten small open reading frames that could encode class II bacteriocins (Hale *et al.*, 2005a; van der Ploeg, 2005). In the present study, the *bsm* genes of the

uncharacterized mutacins were more frequent than the characterized mutacin genes in *Streptococcus mutans*. The frequencies varied between 4 and 95% and the gene expression varied between 2 and 60% among clinical isolates (Fig. 2). The genes *bsm 299* and *bsm 1892c* were more frequently expressed in relation to other bacteriocins. These findings suggest that all putative bacteriocins may represent a large repertoire of inhibitory substances produced by *Streptococcus mutans*. According to van der Ploeg (2005), different combinations of bacteriocins have different antimicrobial spectra.

The clinical isolates expressed between one and seven mutacin biosynthesis genes and were able to inhibit between one and 28 *Streptococcus* spp. indicator strains. There was no association between the mutacin inhibitory spectrum and the number of expressed biosynthesis genes (Spearman correlation test $r=-0.03$; $P>0.05$). In addition, no-mutacin-producing strains expressed between one and four *bsm* genes (Table 6). These results raise several questions: (i) Under what conditions are *bsm* and the characterized mutacin biosynthesis genes expressed and active? (ii) How are they regulated? (iii) How are they secreted?

Gene expression does not imply protein activity. For example, genetic mutations not identified here may modify the proteins and their activities. Low gene expression may hinder protein detection, as at lower levels there will be insufficient antimicrobial activity of the protein and little diffusion in the antagonism assay. However, it is possible that the range of indicator strains selected for this study may not have been sufficiently diverse to detect any activity conferred by different Bsm proteins.

On the other hand, the regulatory or transport mechanisms of these peptides may be defective. Recently, Kreth *et al.* (2006) showed that a group of bacteriocin-like genes (*bsm 423*, *bsm 1906c* and *bsm 1914c*) together with *nlmA* responded strongly to competence-stimulating peptide (CSP) addition, increasing transcription up to 30-fold, suggesting that these genes may form a CSP regulon mediated by the *comDE* two-component regulatory system. Furthermore, according to van der Ploeg (2005), some Bsm proteins depend on transport proteins (ComDE) that also carry the CSP peptide. The presence of this highly conserved region indicates that the expression of these bacteriocin-encoding (*bsm*) genes is under coordinated control by the *comDE* two-component signal transduction system. Another probable transport mechanism, corresponding to NlmTE (ComAB), the *nlmT*-deficient mutant, Δ NlmT, failed to express any inhibitory activity, indicating that the NlmTE ABC transporter is also required for non-lantibiotic export (Hale *et al.*, 2005b).

The phenotypic grouping was not identical to the genotypic grouping. Genotypes with the same qualitative genetic expression profiles did not present the same mutacin production profiles, and distinct genotypes were grouped in the same phenotypic profile of production, showing that the patterns of inhibitory spectra produced by distinct

Table 6. Analysis of expression of biosynthesis genes of mutacins I-IV and Bsm in different bacteriocin-producing phenotypes
Shaded squares, positive expression; open squares, negative expression. Letters from A to Z correspond to the 24 phenotypes (mutacin types).

Producer strain	Genotypic profiles based on expressed genes													Genotype	No. of expressed genes	No. of inhibited strains	Inhibited strains (%)	Phenotypes based on mutacin typing
	<i>mutA</i>	<i>mutAII</i>	<i>mutAIII</i>	<i>nImA</i>	<i>nImB</i>	<i>bsm 283</i>	<i>bsm 299</i>	<i>bsm 423</i>	<i>bsm 1889c</i>	<i>bsm 1892c</i>	<i>bsm 1896</i>	<i>bsm 1906c</i>	<i>bsm 1914</i>					
Sm 12														1	0	23	76.6	[7237711357] (P)
Sm 13															0	23	76.6	[7576734477] (U)
Sm 14															0	21	68.9	[7237711357] (P)
Sm 16															0	13	43.3	[2000027377] (M)
Sm 31															0	2	6.6	[0020400000] (E)
Sm 32															0	2	6.6	[0020400000] (E)
Sm 42															0	0	0	[0000000000] (A)
Sm 43															0	0	0	[0000000000] (A)
Sm 1														2	1	28	93.3	[7777755777] (Z)
Sm 2															1	28	93.3	[7777755777] (Z)
Sm 4															1	27	89.6	[7377755777] (T)
Sm 18														3	1	11	36.6	[0377510000] (J)
Sm 41															1	0	0	[0000000000] (A)
Sm 46															1	0	0	[0000000000] (A)
Sm 23														4	1	5	16.6	[0020100110] (D)
Sm 33															1	1	3.3	[0000200000] (B)
Sm 34															1	1	3.3	[0000200000] (B)
Sm 6														5	1	25	83.3	[3277667377] (O)
Sm 37														6	2	1	3.3	[0000200000] (B)
Sm 22														7	2	8	26.6	[2351000110] (N)
Sm 7														8	2	25	83.3	[7377555777] (T)
Sm 17															2	11	36.6	[0377510000] (J)
Sm 47														9	2	0	0	[0000000000] (A)
Sm 44														10	2	0	0	[0000000000] (A)
Sm27														11	3	3	10.0	[0022200000] (F)
Sm 21														12	3	9	30.0	[0477400000] (K)
Sm 20														13	3	10	33.3	[0376710000] (I)
Sm 8														14	3	25	83.3	[7576734477] (U)
Sm 9															3	25	83.3	[7337713376] (R)
Sm 10															3	25	83.3	[7777717376] (X)
Sm 45														15	3	0	0	[0000000000] (A)
Sm 5														16	4	26	86.6	[7376756777] (S)
Sm 26														17	4	3	10.0	[0022200000] (F)
Sm 3														18	4	27	90.0	[7776737377] (V)
Sm 19														19	4	10	33.3	[0376510000] (H)
Sm 35														20	4	1	3.3	[0000200000] (B)
Sm 36															4	1	3.3	[0000200000] (B)
Sm 40														21	4	0	0	[0000000000] (A)
Sm15														22	5	14	46.6	[0477645022] (L)

Table 6. cont.

Producer strain	Genotypic profiles based on expressed genes													Genotype	No. of expressed genes	No. of inhibited strains	Inhibited strains (%)	Phenotypes based on mutacin typing
	<i>mutA</i>	<i>mutAII</i>	<i>mutAIII</i>	<i>nImA</i>	<i>nImB</i>	<i>bsm 283</i>	<i>bsm 299</i>	<i>bsm 423</i>	<i>bsm 1889c</i>	<i>bsm 1892c</i>	<i>bsm 1896</i>	<i>bsm 1906c</i>	<i>bsm 1914</i>					
Sm 38														23	5	1	3.3	[0000200000] (B)
Sm 11														24	6	23	76.6	[7337613376] (Q)
Sm 24														25	6	3	10.0	[0002500000] (G)
Sm 25														26	7	3	10.0	[0006400000] (C)
Sm 29														27	7	3	10.0	[0006400000] (C)
Sm 30														28	7	3	10.0	[0006400000] (C)
Sm 39															7	1	3.3	[0000200000] (B)
Sm 28														29	7	3	10.0	[0006400000] (C)

Streptococcus mutans isolates are independent of the degree of genetic similarity of the strains tested (Table 6). These results may suggest that the genetic and phenotypic traits of mutacin production are related to the genetic background of each isolate tested, or that genetic polymorphisms or other mutacins are as yet unidentified. In addition, the detection of mutacin biosynthesis gene expression in liquid medium (TSB), in comparison with the inhibitory activities of mutacins detected in solid medium (TSA), may have influenced the lack of relationship between genotypic/phenotypic groupings.

Detailed analysis with purified peptides is necessary to obtain conclusive evidence about the spectrum and role of these antimicrobial proteins. In conclusion, the high diversity of mutacin-producing phenotypes suggests that mutacin production could be an important virulence factor for colonization and establishment of *Streptococcus mutans* in the oral cavity. Furthermore, the high frequency of expression of the biosynthesis genes screened revealed a broad repertoire of genetic determinants encoding anti-microbial peptides that can act in different combinations; however, more studies are necessary to determine the role of each bacteriocin and to explain how mutacin production is regulated and how mutacins are secreted.

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