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In vitro susceptibility of the *Streptococcus milleri* group to antimicrobial peptides

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Abstract

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Aim To determine the susceptibility of strains of the *Streptococcus milleri* group (SMG) to commercially available antimicrobial peptides.

Methodology Thirty strains of SMG from a range of sources were assessed for their susceptibility to 10 antimicrobial peptides of either human, animal or insect origin, using a double layer diffusion assay.

Results The majority of the test strains were sensitive to the amidated peptides, mastoparan (100%; $n = 30$), magainin 2 amide (95%; $n = 21$) and indolicin (91%; $n = 23$). Some strains were susceptible to cecropin B (30%; $n = 30$) and histatin (10%; $n = 30$), whilst no activity was observed for the defensins HNP-1 and HNP-2, histatin 8, cecropin P1 and magainin 2.

Conclusions The majority of strains were resistant to the human derived peptides. The ability to resist such peptides may be a factor in the colonisation of the oral cavity and the survival and initiation of infection in the pulp and root canal environment. Interestingly, the present study indicated that amidated and alpha helical peptides exhibit antimicrobial activity against SMG. Structural modification of these peptides may allow a targeted approach for the development of these substances as preventative or therapeutic agents.

Keywords: antimicrobial peptides, endodontic, infection susceptibility, milleri, streptococcus.

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Introduction

The *Streptococcus milleri* group (SMG) comprises microaerophilic bacteria which are generally regarded as members of the commensal flora of the body. However, SMG are encountered in a variety of infections including those of the oropharynx, cardiovascular, abdominal and central nervous systems (Piscitelli *et al.* 1992, Jacobs *et al.* 1995). Frequently isolated with other bacteria, it has been documented that the SMG are present early in such polymicrobial infections and may actually initiate infection, thereafter preparing the environment for subsequent colonisation by anaerobic species (Lewis *et al.* 1986, 1990, Fisher & Russell 1993). Despite being almost universally susceptible to penicillin, infections involving the SMG are often difficult to treat and are associated with a high morbidity and mortality (Bantar *et al.* 1996).

In the oral cavity, members of the SMG are the most frequent facultative bacteria associated with dentoalveolar abscess and are commonly isolated from necrotic dental pulps and the canals of failed endodontic treatment (Lewis *et al.* 1986, Molander *et al.* 1998, Chavez de Paz *et al.* 2003, Gomes *et al.* 2004, Stefanopoulos & Kolokotronis 2005; Vianna *et al.* 2005). Although endodontic therapy success rates have improved, failure still occurs in an unacceptable proportion of cases and bacteria have been demonstrated in a majority of canals even after treatment with optimal irrigation and mechanical debridement (Basmadjian-Charles *et al.* 2002, Rôças *et al.* 2004, Vianna *et al.* 2006). Agents with more effective anti-

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microbial activity in this environment or ones that may reduce the propensity for remaining bacteria to cause infection would be welcome.

Antimicrobial peptides (AMPs) are an important component of the natural defense system of many living organisms. A wide variety of human peptides have been identified that exhibit broad spectrum activity against Gram-positive and Gram-negative bacteria, yeasts, fungi and enveloped viruses and play an important part in innate immunity (Ganz *et al.* 1985, Hancock *et al.* 1995, Bulet *et al.* 2004). AMPs have also been assigned roles in promoting tissue repair, although their mechanism of action remains unknown (Elsbach 2003). The presence of histatins in saliva, α-defensins in phagocytes and β-defensins produced by epithelial cells and odontoblasts has increased awareness of the likely importance that antimicrobial peptides play in human defense mechanisms and their potential relevance in the pathogenesis and management of infections involving the SMG (Jones & Bevins 1993, Zhao *et al.* 1996, Harder *et al.* 1997, Mizukawa *et al.* 1999, Dommisch *et al.* 2005a,b).

Accordingly, the aim of the study was to examine the efficacy of 10 commercially available antimicrobial peptides, from varying structural groupings, of human and nonhuman origin, against isolates of SMG.

Materials and methods

Bacterial strains and growth conditions

A total of 30 isolates belonging to the SMG was examined (Table 1). The strains were identified as S. *anginosus* (n = 10), S. *constellatus* (n = 10) or S. *intermedius* (n = 10) on the basis of a differential biochemical scheme (Whiley *et al.* 1990) and included the reference strains; S *anginosus* NCTC 10713, S. *constellatus* NCTC 11325 and *S. intermedius* NCTC 11324. Oral strains of SMG originated from either pus samples from dentoalveolar abscesses or plaque specimens obtained from healthy volunteers. Strains isolated from extra-oral sites were kindly provided by R. Whiley (Oral Microbiology, The London Hospital Medical College, London, UK). A lipopolysaccharide (LPS) deficient strain of *Escherichia coli* BUE55 was gifted from R. Dixon (Biomedical Sciences, University of Bradford, UK) and used as a sensitive control organism.

All strains were initially cultured on Columbia agar (Oxoid Ltd., Basingstoke, UK) containing 5% (v/v) horse blood, prior to inoculation into Isosensitest broth (ISB; Oxoid Ltd., Basingstoke, UK) and incubated at 37 °C in an Anaerobic Work Station (Don Whitley Scientific Ltd, Shipley, UK) under an atmosphere of hydrogen 10%, carbon dioxide 10%, nitrogen 80%.

Table 1 Identity and source of 31 test strains used in the present study

Species	Strain reference	Source
S. anginosus	240A/95	Dentoalveolar abscess
S. anginosus	910/95	Dentoalveolar abscess
S. anginosus	322/96	Dentoalveolar abscess
S. constellatus	743/95	Dentoalveolar abscess
S. anginosus	20c/97	Supra-gingival plaque
S. anginosus	10c/97	Supra-gingival plaque
S. anginosus	500/95	Dentoalveolar abscess
S. anginosus	A2940	Blood
S. constellatus	*SOC*	Throat
S. anginosus	7K	Brain abscess
S. anginosus	CDC 2236-81	Blood
S. constellatus	M6561	Appendix
S. anginosus	NCTC 10713	Throat
S. constellatus	313B/95	Dentoalveolar abscess
S. constellatus	428/95	Dentoalveolar abscess
S. constellatus	229/96	Dentoalveolar abscess
S. constellatus	274/96	Dentoalveolar abscess
S. constellatus	322/95	Dentoalveolar abscess
S. constellatus	4515/96	Perianal abscess
S. constellatus	5′c/97	Sub-gingival plaque
S. constellatus	R87/3795	Blood
S. constellatus	C1792	Spinal osteomyelitis
S. constellatus	NCTC 11325	Pleurisy
S. intermedius	240B/95	Dentoalveolar abscess
S. intermedius	447/95	Dentoalveolar abscess
S. intermedius	127/96	Dentoalveolar abscess
S. intermedius	313A/95	Dentoalveolar abscess
S. intermedius	28c/97	Sub-gingival plaque
S. intermedius	23′c/97	Sub-gingival plaque
S. intermedius	HW69	Brain abscess
S. intermedius	R87/3972	Blood
S. intermedius	F458L	Abdominal mass
S. intermedius	NCTC 11324	Unknown
E. coli	BUE55	Unknown

Peptides

Amino acid sequences of the peptides obtained commercially (Sigma, Poole, UK) are shown in Table 2. Stock solutions of the defensins, human neutrophil peptides (HNP 1 and 2), and the histatins were dissolved in 0.01% (v/v) acetic acid and the remainder were dissolved in 0.1 remainder were dissolved in 0.1 M phosphate buffer, pH 6.4. The peptide solutions were tested at concentrations of 1 mM with the exception of the defensins which were prepared as 500 μM solutions. All the peptide solutions were stored at −87 °C.

Table 2 Source, structure and sequence of 10 commercially available peptides

Peptide	Source	Structure	Sequence
Human neutrophil peptide 1 (HNP 1)	Human neutrophil	3 sheet	A-C-Y-C-R-I-P-A-C-I-A-G-E-R-R-Y-G-T-C-I-Y-Q-G-R-L-W-A-F-C-C
Human neutrophil peptide 2 (HNP 2)	Human neutrophil	3 sheet	C-Y-C-R-I-P-A-C-I-A-G-E-R-R-Y-G-T-C-I- Y-Q-G-R-L-W-A-F-C-C
Histatin 5	Human saliva	Weak a helix	D-S-H-A-K-R-H-H-G-Y-K-R-K-F-H-E-K-H-H-S-H-R-G-Y
Histatin 8	Human saliva	Weak a helix	K-F-H-E-K-H-H-S-H-R-G-Y
Indolicidin	Bovine granulocyte	Weak extended helix	I-L-P-W-K-W-P-W-W-P-W-R-R-NH2
Cecropin B	Silk moth haemolymph	A helix	K-W-K-V-F-K-K-I-E-K-M-G-R-N-I-R-N-G-I-V-K-A-G-P-A-I-A-V-L-G-E-A-K-A-L-NH2
Cecropin P1	Pig intestine	A helix	S-W-L-S-K-T-A-K-K-L-E-N-S-A-K-K-R-I-S-E-G-I-A-I-A-I-Q-G-G-P-R
Magainin II	Frog intestine and skin	A helix	G-I-G-K-F-L-H-S-A-K-K-F-G-K-A-F-V-G-E-I-M-N-S
Magainin II (Ala 8,13,18) amide	Frog intestine and skin	A helix	G-I-G-K-F-L-H-A-A-K-K-F-A-K-A-F-V-A-E-I-M-N-S-NH2
Mastoparan	Wasp venom	A helix	I-N-L-K-A-L-A-A-L-A-K-K-I-L-NH2

A, alanine; C, cysteine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan; Y, tyrosine.

Assay procedure

The antimicrobial activity of the peptides was assessed by a modification of a previously described radial diffusion assay (Lehrer *et al.* 1991). Bacterial strains were cultured in ISB to exponential phase and adjusted to an optical density at 540 nm corresponding to 2×10^6 colony forming units (cfu) mL^{-1}. Molten agar (50 °C) containing 12 mL of half strength ISB plus 1% (w/v) low electro-endosmotic agarose (Sigma, Poole, UK) and 0.02% Tween 20 (Sigma, Poole, UK) was inoculated with 100 µl of the adjusted bacterial suspension. Peptide test solutions (2 p1) were added to 1.5 mm wells cut into the agar using sterile Pasteur pipettes. Acetic acid 0.01% (v/v) and 0.1 M phosphate buffer pH 6.4 served as negative controls. Following a 3 h incubation at 37 °C to allow diffusion of peptides, a 12 mL overlay of molten (50 °C) double strength ISB containing 1% (w/v) agarose (Sigma, Poole, UK) was added. The plates were incubated for 18 h at 37 °C in an anaerobic atmosphere. Resultant zones of growth inhibition were measured with a ruler using a colony counter under ×1.5 magnification. Sensitivity was recorded as the presence of a clear zone of 2.5 mm or greater (Moore *et al.* 1996).

Results

Susceptibility of SMG strains to the different antimicrobial peptides varied according to the type of peptide (Table 3). At 1 mM, indolicidin, magainin II amide and mastoparan inhibited the growth of the majority of test isolates with 91% (n = 23), 95% (n = 21) and 100% (n = 30) of strains respectively being sensitive. A smaller proportion of the strains was susceptible to histatin 5 (10%, n = 30) and cecropin B (30%, n = 30). The defensins (HNP 1 and 2), histatin 8, cecropin P1 and magainin II did not inhibit the growth of any of the strains.

All the SMG isolates tested gave a similar sensitivity profile to the antimicrobial peptides under investigation, with no obvious differences in peptide susceptibility between the three species, S. *anginosus*, *S. constellatus* and S. *intermedius* regardless of the clinical source of the strains.

The largest zones of growth inhibition of the SMG were observed with mastoparan and magainin II amide having a similar mean diameter of 8.9 mm (+/−2.8) and 8.8 mm (+/−2.4) respectively. Smaller zones of growth inhibition were noted with indolicidin (6.0 mm, +/−2.4), cecropin B (5.1 mm, +/−1.2) and histatin 5 (3.9 mm, +/−0.9).

The LPS deficient mutant strain, E. coli BUE55 was sensitive to six of the antimicrobial peptides under investigation and resistant to the defensins and histatins. With three peptides, the zone sizes of the control strain were greater than those exhibited against the SMG strains. Cecropin B produced a mean growth inhibition zone of 22.3 mm (+/−1.3), magainin II amide, 19 mm and mastoparan a 11.5 mm (+/−1.5)

Table 3 Sensitivity of 30 strains of SMG to 10 antimicrobial peptides

Peptide[a]	Number of strains tested	Number of strains sensitive	(%)	*E. coli* BUE55
Human nutrophil peptide 1	30	0	0	R[b]
Human nutrophil peptide 2	29	0	0	R
Histatin 5	30	3	10	R
Histatin 8	30	0	0	R
Indolicidin	23	21	91	S[c]
Cecropin B	30	9	30	S
Cecropin P1	13	0	0	S
Magainin II	30	0	0	S
Magainin II amide	21	20	95	S
Mastoparan	30	30	100	S

[a]1 mM except defensins (HNP 1 and 2) at 500 μM.
[b]R = resistant, zone of growth inhibition <2.5 mm.
[c]S = sensitive, zone of growth inhibition ≥2.5 mm.

zone. The growth inhibitory response of the control strain was similar to the SMG isolates with indolicidin producing a clear zone of 5.9 mm (+/−1.2). In contrast to the SMG strains, the E. coli strain was susceptible to the antibacterial actions of magainin 1110.3 mm (+/−2.5) and cecropin P 1 19.0 mm (+/−1.4).

Whilst not producing a clear zone of growth inhibition that was consistent with sensitivity, some isolates exhibited a zone of partial growth inhibition to certain peptides; cecropin B (14 strains), HNPI (nine strains), histatin 5 (eight strains), HNP2 (five strains) and magainin II (four strains). These zones occurred with peptides capable of limited inhibitory activity and were generally smaller in diameter than those of the fully sensitive strains.

Discussion

In the present study a wide range of antibacterial activity towards SMG isolates was seen depending on the peptide being assayed. The test peptides demonstrated either no antimicrobial activity, partial growth inhibition response or a zone of complete inhibition of growth. The diffuse zones observed in some instances may represent a region of delayed growth due to a bacteriostatic response or a sub-population within the strain that has an intermediate resistance to the peptide. This response has been documented in a similar agar plate assay using a strain of methicillin resistant *S. aureus* (MRSA; Helmerhorst *et al.* 1997) and the sensitive strain of *E.* coli used here (Moore *et al.* 1996). In the present study such zones, containing minute colonies of SMG, were recorded as resistant regardless of the size of the zone.

The synthetic peptides of human origin (defensins and histatins) investigated showed no or poor activity against SMG or against the control strain of *E. coli.* Only three strains of SMG were susceptible to histatin 5, whilst growth of none of the isolates was inhibited by histatin 8. A further eight strains of SMG showed only a diffuse zone of growth inhibition with histatin 5. Previous work has demonstrated that histatins have growth inhibitory effects on *Streptococcus mutans* (MacKay *et al.* 1984). The lack of significant activity of the histatins against SMG observed here is in agreement with the findings of another recent study that reported no bactericidal activity of histatin 5 against *S. mutans* and other oral bacteria (Helmerhorst *et al.* 1997).

The low activity of the cecropins against SMG is consistent with previous reports that found that cecropins have greater activity against Gram-negative bacteria (Lee *et al.* 1989, Moore *et al.* 1996). Although cecropin B was shown to inhibit the growth of nine SMG strains, no activity was observed for cecropin P1. Growth of a number of SMG strains (14/30), was weakly inhibited by cecropin B as evidenced by the partial zones.

Magainin II amide, mastoparan and indolicidin possess an amidated carboxy terminus and the high level of activity of these peptides suggests that the possession of this property may in part be responsible for bactericidal activity against SMG strains. Furthermore, peptides without the amidated group, (cecropin PI and magainin 11), were ineffective at inhibiting the growth of SMG in contrast to the similar amidated molecules, cecropin B and magainin II amide. Amidation of the carboxy-terminal has been shown to increase the spectrum of activity of the cecropins especially against Gram-positive bacteria (Li *et al.* 1988). Improvements in the bactericidal potencies, resistance to proteolysis, cytotoxicity and in the spectrum of activity have been achieved producing peptide

analogues with selected amino acid changes, small repeats of peptides and hybrid molecules (Raj *et al.* 1990, Bessalle *et al.* 1993, Zuo *et al.* 1995, Ramalingam *et al.* 1996, Falla & Hancock 1997). Many of these analogues have a stable amphipathic helical structure which can be correlated to the antibacterial activity. The low helical propensity residues in magainin 11 were substituted with alanine to improve the amphipathic helical formation to produce magainin II amide (Ala8, 13,18; Chen *et al.* 1998). In the present study, this analogue of magainin II amide was highly effective at inhibiting the growth of SMG isolates. The shorter alpha helical and amidated peptides appear to possess antibacterial activity against SMG strains. Structural and functional studies have indicated the importance of the positioning of positive charges, amphiphilicity, alpha helicity and hydrophobicity in the modulation of antibacterial activity (Lee *et al.* 1986, Bessalle *et al.* 1993, Wieprecht *et al.* 1997).

Members of SMG are noted for their heterogeneity both genetically and phenotypically (Jacobs *et al.* 1995; Whiley & Hardie 1989, Whiley *et al.* 1990, 1995, 1997) and therefore the relatively uniform response of the isolates to the antimicrobial peptides observed in the present study was unexpected. It has been reported that SMG display a wide range of pathogenic determinants and variation in their cell-surface associated properties, although most strains carry a net negative charge with varying hydrophobicities and surface charge (Willcox & Knox 1990). The nature of the bacterial cell surface has an important influence on the susceptibility to antimicrobial peptides (Maloy & Kari 1995). The mode of action of cationic antimicrobial peptides is thought to involve an initial electrostatic interaction with the negatively charged target cell surface molecules. This interaction is followed by a conformational change which leads to the disruption of the cell membrane either by the formation of channels spanning the membrane or by a global disturbance of the lipid bilayer. The similar susceptibility profile to antimicrobial peptides observed here suggests that the cell surface of SMG share a number of common features.

The present study investigated the antibacterial activity of a variety of peptides against a number of isolates from SMG, in contrast to previous studies which have been limited to representative strains of a spectrum of bacterial species (Cullor *et al.* 1990, Gunshefski *et al.* 1994, Fernandez & Weiss 1996, Halling 1996, Yasin *et al.* 1996). The results presented here show that the helical and amidated peptides magainin II amide, mastoparan and indolicidin are highly effective in inhibiting the growth of members of SMG.

The disc diffusion method employed has obvious limitations including the study of pure cultures of bacteria in planktonic state. It would be interesting to investigate the use of other methods such as poloxamer hydrogels for susceptibility testing. It has been demonstrated that many bacteria exist in a true biofilm state within such gel systems (Cluttterbuck *et al.* 2007) and it is possible that this method could be modified to study mixed populations, thus more closely mimicking the *in vivo* situation of a complex biofilm within the root canal. If the peptides are shown to be active under such conditions their activity following the incorporation into root canal dressings and possibly sustained release material systems would then be assessed. Advantages of peptides over other commonly used antimicrobial agents include their selectivity and the fact that the development of resistance to these agents is thought to be relatively rare.

The discovery of additional antimicrobial peptides in humans has increased our understanding regarding the role that these agents play in the host defence (Mallow *et al.* 1996, McCray & Bentley 1997). The resistance of the SMG to the human antimicrobial peptides in particular may confer a selective advantage on the survival of the micro-organisms *in vivo* (Groissman *et al.* 1992) and may be an important factor in the determination of bacterial pathogenicity. Changes in the design of these bio-active peptides, to improve their antibacterial activity and lower their cytotoxicity, may in the future allow them to developed as therapeutic agents in the treatment and prevention of SMG infections including those of pulpal tissues.

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