Prevention of streptococcal pharyngitis by anti-*Streptococcus pyogenes* bacteriocin-like inhibitory substances (BLIS) produced by *Streptococcus salivarius*

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Background & objectives: *Streptococcus salivarius* is a numerically prominent member of the human oral microbiota that produces a variety of bacteriocin-like inhibitory substances (BLIS) having *in vitro* inhibitory activity against *S. pyogenes*. Our previous studies of *S. salivarius* isolates from children using a deferred antagonism BLIS production (P)-typing scheme showed that the 9 per cent of children having large populations of P-type 677 *S. salivarius* experienced fewer *S. pyogenes* acquisitions than either the 11 per cent of children having predominant P-type 226 populations or the 60 per cent of children with largely non-inhibitory (P-type 000) *S. salivarius*. Amongst the other BLIS P-types detected were a number of strongly-inhibitory (P-type 777) *S. salivarius*. In the present study the inhibitory agents produced by prototype strains of P-types 226, 677 and 777 *S. salivarius* are compared.

Methods: The prototype BLIS-producing *S. salivarius* strains SN, 20P3, and K12 were isolated from tongue swabblings. BLIS P-typing was done using standard procedures. The BLIS molecules were purified and characterized.

Results: *S. salivarius* SN (P-type 226) produces a heat-labile muramidase. *S. salivarius* 20P3 (P-type 677) produces the 2315 Da lantibiotic salivaricin A and *S. salivarius* K12 (P-type 777) produces two lantibiotics; salivaricin A2 (2368 Da) and salivaricin B (2733 Da).

Interpretation & conclusion: The P-type 777 *S. salivarius* strain produced salivaricin A2 and salivaricin B. The combined production of two anti-*S. pyogenes* BLIS activities by this strain indicates that it could be adopted as a colonizing strain in bacterial interference trials.

Key words Bacteriocin - bacteriocin-like inhibitory substance - lantibiotic - replacement therapy - *Streptococcus salivarius*

*Streptococcus pyogenes* is a major cause of acute infections and of serious delayed sequelae, particularly in children. At present, the most effective strategy available to deal with *S. pyogenes* is treatment of acute infections by administration of therapeutic doses of a broad-spectrum antibiotic like penicillin. Also, since there is currently no anti-*S. pyogenes* immunization available, the only means of protecting at risk individuals is antibiotic prophylaxis. However, these approaches have many inherent problems: the cost of the antibiotics, the possibility of adverse host reactions, severe disruption of the indigenous microbiota and bacterial resistance development. The implementation of bacterial interference may offer a relatively specifically-targeted alternative means of preventing the development of acute *S. pyogenes* infections. Since commensal streptococci are numerically predominant in the oral cavity, it seems reasonable to speculate that they may be central to any naturally occurring interference with colonization or infection by *S. pyogenes*. Children who acquire
*S. pyogenes* have been shown to have a lower proportion of throat cultures containing bacteria inhibitory or bacteriocidal for *S. pyogenes* than those who do not become colonised. The pantothenic acid antagonist enocin, produced by *S. salivarius*, was later speculated to contribute to protection against *S. pyogenes* infections. Grahn and Holm established that the prevalence of oral alpha-haemolytic streptococci having inhibitory activity against *S. pyogenes* was lower in those children who became infected during an outbreak of streptococcal tonsillitis. This group later recommended dosing with a mixture of alpha haemolytic streptococci as a supplementary treatment of recurrent streptococcal tonsillitis.

Of all the bacterial species known to regularly inhabit the human oral microbiota in large numbers *S. salivarius* is perhaps the most innocuous. There appear to be no reports of this species causing infections within the oral cavity and the rare instances of its association with bacteraemia or meningitis appear to have occurred in immunologically-compromised patients or following trauma to the patients tissues. Since *S. salivarius* is common, not only on the dorsum of the tongue but also on the oropharyngeal mucosa, it is well positioned to directly repel invasion by *S. pyogenes*. Our previous studies have demonstrated that approximately 45 per cent of *S. salivarius* strains inhibit the growth of one or more members of a set of 9 strains used routinely as indicators of streptococcal BLIS. The pattern of inhibition of these indicators, when expressed in code form, is referred to as the BLIS production (P)-type of a bacterium. Subsequent studies of streptococcal pharyngitis in populations of school-aged children showed that some children were seldom infected. Many of these rarely-infected children were found to harbour large populations of *S. salivarius* producing anti-*S. pyogenes* BLIS activity. A study of 780 Dunedin school children found that there were two major types of BLIS activities produced by the *S. salivarius*, the corresponding P-type patterns being referred to as 226 (11% of children positive) and 677 (9% positive). A further 20 per cent of the children had *S. salivarius* of various other P-type designations, including some isolates producing particularly strong (P-type 777) BLIS activity. The present study compares the BLIS activities produced by *S. salivarius* strains SN, 20P3 and K12, the prototypes of BLIS P-types 226, 677 and 777 respectively.

### Material & Methods

**Bacterial strains and BLIS P-typing:** The prototype BLIS-producing *S. salivarius* strains SN (P-type 226), 20P3 (P-type 667) and K12 (P-type 777) were isolated on Mitis Salivarius agar (Difco Laboratories, Detroit) from tongue swabblings of human subjects. The 9 standard indicators and the standard procedure used for the BLIS P-typing of streptococci have been described previously.

**Isolation and characterization of BLIS molecules:** The BLIS produced by *S. salivarius* strain SN was purified from 18 h 35°C Todd Hewitt broth (Difco) culture supernatants using the procedures previously devised for the similar BLIS, zoocin A, produced by *S. equi* subsp. *zooepidemicus*. The procedures for isolation of the lantibiotic peptides salivaricin A2 and salivaricin B from *S. salivarius* strain K12 were those applied previously to the purification of salivaricin A from *S. salivarius* strain 20P3. Amino acid compositions, mass spectrometry and N-terminal amino acid sequencing were done by the Protein Microchemistry Facility, Department of Biochemistry, University of Otago. To enable Edman degradation to proceed through blockages caused by the presence of dehydro amino acids, these residues were first modified by addition of thiol groups.

### Results

A muralytic enzyme, salivaricin SN, was isolated from the supernatants of Todd Hewitt broth cultures of *S. salivarius* strain SN. Tests of the inhibitory spectrum of strain SN using the deferred antagonism test established that 18 of 20 *S. pyogenes* were susceptible, but none of a variety of other streptococci, including representatives of Lancefield groups B, C, D, F and G, *S. salivarius*, *S. sanguinis* and *S. mutans*. The purified protein was inactivated on heating at 80°C for 30 min. The N-terminal sequence of salivaricin SN was DINGGANTPGAYD.

*S. salivarius* strain 20P3 has been shown previously to produce the lantibiotic salivaricin A (Table). Also shown in the Table is the sequence of salivaricin A1, the homologous lantibiotic produced by the serotype M4 *S. pyogenes* strain 2006.
S. salivarius strain K12 was found to produce two anti-S. pyogenes peptides (Table). The detection of lanthionine in amino acid analyses and stability to heating at 100°C for 30 min indicated that both are of the lantibiotic class of bacteriocins. One of these was a T4S/F7I variant of salivaricin A, named salivaricin A2. The second was quite unrelated to salivaricin A and is referred to as salivaricin B.

Discussion

The present study shows that the anti-S. pyogenes inhibitory activities previously shown to be relatively commonly produced in vitro by S. salivarius are of three markedly different molecular types. Strain SN, the prototype of the P-type 226 S. salivarius produced a heat labile lytic enzyme, salivaricin SN, the N-terminus of which was completely different from that of the previously-described streptococcal muralysin, zoocin A (ATYTRPLDTG…). Zoocin A has been shown to be a domain-structured enzyme similar to lysostaphin, with the N-terminus responsible for catalysis and the C-terminal domain effecting target recognition15. Unlike zoocin A, which displays strong activity against S. mutans in addition to S. pyogenes, the S. salivarius agent appears relatively specifically active against S. pyogenes. Cloning of the salivaricin SN structural gene is in progress and when completed will facilitate direct comparison between the two enzymes for evidence of domain sharing.

Our previous studies13,14 have shown that the structural gene salA, which encodes the lantibiotic salivaricin A, is the usual form detected in S. salivarius. PCR amplification and sequencing of the salA-like gene in 15 probe-positive S. pyogenes revealed consistent codon differences indicative of conservative amino acids changes in positions 2 (K instead of R) and 7 (F instead of I) of the salivaricin A propeptide. This variant form, called salivaricin A1, appears to be expressed as a functional product only in serotype M4 strains of S. pyogenes but is sufficiently similar to effect induction of salivaricin A production in salA-positive strains of S. salivarius.

In the present study, yet another variant of salivaricin A has been found to be produced by S. salivarius strain K12. Interestingly this variant, salivaricin A2, which differs in two conservative amino acid substitutions from both salivaricin A and salivaricin A1, appears commonly to be produced together with salivaricin B in P-type 777 S. salivarius (data not shown). The P-type 777 S. salivarius strain K12 was shown to produce in addition to salivaricin A2 an unrelated lantibiotic salivaricin B. The combined production of two anti-S. pyogenes BLIS activities by strain K12 was one consideration leading to its adoption as a colonizing strain in bacterial interference trials in populations of school children and young adults.

Acknowledgment

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References


### Table. Primary sequence and molecular mass of lantibiotic salivaricins

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<th>Salivaricin</th>
<th>Amino acid sequence</th>
<th>Mass (Da)</th>
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<tr>
<td>A*</td>
<td>KRGSGWIAITDDCPNSVFVCC</td>
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</tr>
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* The data for salivaricin A10 and salivaricin A113 have been previously published


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