Production and characterization of salivaricin MPS-like inhibitory activity from *Streptococcus uberis* strain NY42

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Dedicated to my parents

獻給我的父母
Abstract

*Streptococcus salivarius*, a predominant bacterial species found in the human oral cavity, is increasingly studied for its production of bacteriocin-like activity. Much of the interest in the study of *S. salivarius* is driven by the perceived potential probiotic applications. The preliminary characterization of salivaricin MPS (salMPS), a bacteriocin produced by *S. salivarius* strain MPS revealed a large antimicrobial molecule with specific inhibitory activity against *Streptococcus pyogenes*, a potential human pathogen.

In the present study, the production of salivaricin MPS was evaluated from its original producer strain MPS using various growth media. Contrary to previous findings, it was almost impossible to detect any inhibitory activity when the producer was grown in liquid THB (Todd-Hewitt broth) medium, with or without supplement. The additional inhibitory activity of strain MPS towards non-*S. pyogenes* indicators and presumed two-component inhibitor system (from two salMPS genes) further complicated the production scenario. In comparison, *Streptococcus uberis* strain NY42 which was selected from the screening study as an alternative salMPS producer demonstrated a more specific anti-*S. pyogenes* salMPS-like inhibitor activity while containing only one of the two salMPS genes found in strain MPS.

Recovery of salMPS-like activity from strain NY42 was mainly achieved by freeze thaw method extraction of cultures grown on blood agar. The biggest challenge during the enrichment of the inhibitory activity using ammonium sulfate precipitation was the interference by large amounts of haemoglobin from the blood agar that associated with the inhibitory activity. Attempts at removing the haemoglobin from the inhibitory activity using guanidine hydrochloride and urea did not succeed. Fractionation of the NY42 freeze thaw sample using gel permeation HPLC (high pressure liquid chromatography) did indicate the presence of the putative salMPS-like inhibitor
within the active fractions, supported by the SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) and mass spectrometry analysis results, although data from online database searching was less significant due to the lack of suitable library entries.

The effect of blood and saliva as well as uv-irradiation on the production of inhibitory activity by several P-type 226 producer strains was also investigated in this study. In addition, the possibility of using \emph{S. pyogenes} cells to bind salMPS-like activity, followed by an attempt to release the inhibitory activity was also assessed.

Despite all the difficulties with the original producer \emph{S. salivarius} strain MPS, the study of strain NY42 discovered an alternative producer capable of producing a similar but more specific inhibitory activity compared to strain MPS. Purification of salMPS-like inhibitor using HPLC, combined with 1D (one dimension) -PAGE and mass spectrometry analysis further demonstrated the possibility of using strain NY42 as a favorable salMPS producer.
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## Table of content

Abstract ...................................................................................................................................i

Acknowledgement ................................................................................................................iii

Table of content ....................................................................................................................iv

List of Tables ........................................................................................................................vii

List of Figures .......................................................................................................................viii

Abbreviations .......................................................................................................................x

Chapter 1 Introduction ............................................................................................................1

1.1 Bacteriocins of Gram-negative bacteria ..........................................................................2

1.2 Bacteriocins of Gram-positive bacteria ..........................................................................3

1.2.1 Class I: Lanthionine-containing bacteriocins .................................................................4

1.2.1.1 Nisin – an example of type A lantibiotics .................................................................4

1.2.1.2 Type B and type C lantibiotics ................................................................................7

1.3 Class II and Class IV bacteriocins ................................................................................8

1.4 Class III bacteriocins and salivaricin MPS ...................................................................10

Chapter 2 Materials and Methods .......................................................................................13

2.1 Salivaricin MPS producer strains and indicator strains ..................................................13

2.2 Bacterial growth media and cultures ..............................................................................14

2.3 Sterilization and storage conditions ................................................................................15

   2.3.1 Sterilization and storage of growth media .................................................................15

   2.3.2 Chloroform sterilization ..............................................................................................15

   2.3.3 UV irradiation sterilization ........................................................................................15
2.4 Salivaricin MPS Production from S. salivarius Strain MPS........................16
  2.4.1 Salivaricin MPS production in modified liquid medium......................16
  2.4.2 Salivaricin MPS production in bi-phasic medium...............................16
2.5 Tests for Inhibitory Activity............................................................17
  2.5.1 Simultaneous antagonism test.........................................................17
  2.5.2 Deferred antagonism test...............................................................17
  2.5.3 Well diffusion and spot diffusion assays.........................................17
2.6 Polymerase Chain Reaction (PCR) Screening for Potential Producers of
  salMPS-like Activity................................................................................18
2.7 Extraction of prototype salMPS inhibitor from BACa agar cultures………19
  2.7.1 Freeze-thaw extraction and urea extraction of salMPS-like inhibitory
       agents...............................................................................................19
  2.7.2 Attempted removal of haemoglobin from extractions using urea and
       guanidine hydrochloride (GuHCl).........................................................20
  2.7.3 Ammonium sulfate precipitation.......................................................21
  2.7.4 Adsorption to and elution of inhibitory agents from viable or from
       heat-killed S. pyogenes cells...............................................................21
2.8 Gel permeation HPLC of dialysed crude Ny42 preparations.................22
  2.8.1 SDS-PAGE of inhibitory fractions from the dialysed crude NY42
       preparation...........................................................................................23
2.9 Ion-Exchange Chromatography............................................................24

Chapter 3 Results and Discussion.........................................................26

  3.1 Salivaricin MPS production by Streptococcus salivarius strains MPS......26
    3.1.1 Salivaricin MPS production using solid growth media.....................26
    3.1.2 Salivaricin MPS production in a modified liquid growth medium.....30
    3.1.3 Salivaricin MPS production using bi-phase medium.......................34
3.2 Studies of salivaricin MPS-like inhibitory activity produced by other streptococcal strains

3.2.1 Screening of streptococci for salivaricin MPS-like anti-pyogenes activity

3.2.2 Factors potentially influencing the production and detection of salivaricin MPS-like activity

3.3 Attempt to adsorb the prototype salMPS inhibitor to target cells

3.4 Purification of prototype salMPS inhibitor from strain NY42 using HPLC

3.4.1 Inhibitory activity of ammonium sulfate fractionated material

3.4.2 Gel permeation HPLC of precipitated strain NY42 freeze thaw extract

3.4.3 1D-SDS-PAGE of inhibitory fractions from gel permeation HPLC

3.4.4 Mass spectrometry results of inhibitor-positive gel permeation HPLC fraction on SDS-PAGE

3.4.5 Attempts to remove the haemoglobin content of the freeze thaw extract samples using urea and GuHCl

3.5 Preliminary ion-exchange chromatography fractionation of NY42 extract containing inhibitory activity

3.5.1 Ion-exchange column binding test of NY42 freeze thaw extract

3.5.2 Fast protein liquid chromatography

Chapter 4 Conclusions

References
List of Tables

Chapter 2 Materials and Methods

Table 2.1.1 Standard Indicators.................................................................14

Table 2.9.1 Ion-exchange columns and buffer selection..............................24

Chapter 3 Results and Discussion

Table 3.1.1.1 P-types of salivaricin MPS-producing *S. salivarius* strains MPS and Mia under aerobic and anaerobic growth conditions.................................26

Table 3.1.2.1 Production of salivaricin MPS-like activity by *S. salivarius* strains MPS and Mia using liquid growth media.......................................................33

Table 3.1.3.1 Production of salivaricin MPS-like activity by *S. salivarius* strains MPS and Mia using bi-phase medium.........................................................36

Table 3.2.1.1 The salMPS and salMPSvar content and P-types of potential producers of salivaricin MPS-like activity.........................................................39

Table 3.4.4.1 Database search results of MS/MS sequences from the SDS-PAGE gel..............................................................59
List of Figures:

Chapter 3 Results and Discussion

Figure 3.1.1.2 P-typing and simultaneous antagonism tests of *S. salivarius* strains Mia and MPS.................................................................29

Figure 3.2.1.2 Extraction of inhibitory activity from various *S. salivarius* strains and *S. uberis* strain NY42 using 7 M urea.........................................................40

Figure 3.2.2.1 The effect of UV, blood and saliva on the inhibitory activity of *S. salivarius* strain MPS.................................................................44-45

Figure 3.4.1.1 Activity of NY42 freeze thaw extract after 50% ammonium sulfate fractionation.................................................................50

Figure 3.4.2.1 Gel permeation HPLC chromatography of ammonium sulfate fractionated NY42 BACa agar freeze thaw extract material.........................52

Figure 3.4.2.2 Activity of HPLC-fractionated ammonium sulfate precipitated freeze thaw extract of *S. uberis* NY42.................................................................54

Figure 3.4.3.1 SDS-PAGE of gel permeation HPLC active fractions of strain NY42 freeze thaw extract.................................................................56

Figure 3.4.5.1 Dissociation of haemoglobin by GuHCl...........................................61
Figure 3.5.1 Inhibitory activity of desalted NY42 freeze thaw extract after ammonium sulfate precipitation

Figure 3.5.1.1 Inhibitory activity analysis of preliminary ion-exchange fractionation

Figure 3.5.2.1 (a) FPLC of the CM column effluent material from the column binding test

Figure 3.5.2.1 (b) FPLC of desalted 80% ammonium sulfate precipitated NY42 freeze thaw extract
Abbreviations

1D  one dimension
ABC  ATP-binding cassette
AMP  antimicrobial peptides
AU  activity unit
BACa  blood agar medium with 0.5 % (w/v) calcium carbonate
BLIS  bacteriocin-like inhibitory substance
°C  degree Celsius
CAB  Columbia agar base
CaCO₃  calcium carbonate
cm  centimeter
CM  carboxymethyl
CDM  chemically defined medium
CO₂  carbon dioxide
Cys  cysteine
DEAE  diethylaminoethyl
FPLC  fast protein liquid chromatography
g  gram(s)
g (italic)  gravitational force
GuHCl  guanidine hydrochloride
HPLC  high performance liquid chromatography
<table>
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<td>kilobase</td>
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<td>L</td>
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<tr>
<td>LAB</td>
<td>lactic acid bacteria</td>
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<tr>
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<tr>
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<tr>
<td>MS</td>
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<td>sodium dodecyl sulphate</td>
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<td>Symbol</td>
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<td>------------------------------</td>
</tr>
<tr>
<td>s</td>
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</tr>
<tr>
<td>salMPS</td>
<td>salivaricin MPS</td>
</tr>
<tr>
<td>THB</td>
<td>Todd-Hewitt Broth</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
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<td>volume(s)</td>
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<td>milliamp</td>
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Human bodies can be colonised by a wide array of microorganisms, forming distinctive microflora at various locations. Among all of these microorganisms, bacteria are the most common and well-known inhabitants, and have been increasingly studied regarding their relationship with human health, along with their interaction with other co-existing bacterial species living in the same vicinity. The antimicrobial activity of one bacterium towards another forms the basis of bacterial antagonism. Such interference between bacteria of different species could prove to be beneficial to the host organism, under a scenario where the growth of one particular pathogenic species is inhibited by the antagonistic action of bacteria of another non-pathogenic species (Dodd, 1999). Bacterial antagonism is commonly initiated by one bacterium producing an anti-microbial molecule which acts on members of its target species. One subset of such molecules is called the bacteriocins and unlike other antibiotics, bacteriocins are ribosomally-synthesized proteinaceous molecules that are released extracellularly by bacteria. Depending on the producing bacterium, the activity spectrum of a bacteriocin usually includes other bacteria which are genetically closely-related to the producing species. However, most producer strains are not inhibited by their own bacteriocin, due to expression of specific immunity gene(s) (Heng et al., 2007).

This present study is focused on the characterization of a particular group of bacterial strains from several Streptococcus species including Streptococcus salivarius and Streptococcus uberis, all of which are capable of producing a Streptococcus pyogenes-specific inhibitory activity, similar to salivaricin MPS (salMPS). Salivaricin MPS has previously been described and partially characterized as a macro-bacteriocin produced by Streptococcus salivaricus strain MPS and also by strain
Mia, that shows a specific inhibitory pattern against *S. pyogenes* (Dodd, 1999), a pathogenic species commonly found in the human oral cavity.

### 1.1 Bacteriocins of Gram-Negative Bacteria

The studies of inter-bacterial inhibition can be traced back to the work done by Gratia in the 1920s, who described the antagonism between strains of *Escherichia coli*, a gram-negative bacterium (Gratia, 1925). The discovery and subsequent characterization of several colicins, the first documented bacteriocins produced by members of *E. coli*, revealed more details of their biochemical structures and killing mechanisms (Heng *et al.*, 2007).

Colicins are large multi-domained, plasmid-encoded bacteriocins (Håvørstein *et al.*, 1994). Their biology shows some similarity to typical bacteriocins produced by Gram-positive bacteria. Colicin synthesis is repressed by the SOS response, an inducible DNA repair system which in many bacteria, helps them survive under DNA-damaging circumstances (Michel, 2005). The release of colicin into the extracellular medium involves small lipoproteins, which usually contain 25 to 35 amino acids; these proteins are also known as colicin lysis proteins, as apart from promoting colicin release, they also provoke modifications of the cell envelope (Cascales *et al.*, 2007).

The killing pathway of most colicins is mediated through three domains in a sequential pathway. The binding of a central domain to a specific receptor is the first step of colicin activation. This binding domain is located within the central region of all colicins (Brunden *et al.*, 1984). The N-terminal domain helps colicins to translocate across the outer membrane of their target cells. Unlike the central and C-terminal domain, the N-terminal domains of different colicins display less
similarity. Variation in colicins depends on which import system (Tol or Ton) is used (Pilsl et al., 1995). The killing activity of colicins is carried out by the C-terminal domain, by either producing pore(s) in target cell membranes, or by cutting up the genetic material (DNA or RNA) of the target cell via nuclease activity (Bullock et al., 1983; Martinez et al., 1983).

The recent study of colicin Ia by Greig and colleagues, revealed more details of the channel-forming mechanism in sensitive *E. coli* cells. By generating a two-dimensional crystal of colicin Ia complexed with a lipid-bilayer membrane, it was found that instead of forming a voltage-gated ion channel and resulting cell death by a single colicin Ia molecule in the conventional three-step pathway, more molecules were involved in the channel architecture, and may be responsible for large scale peptide translocation upon channel opening (Greig et al., 2009).

1.2 Bacteriocins of Gram-positive Bacteria

As more microbiologists shifted their focus to Gram-positive bacteria, the number of bacteriocins discovered from this group has been growing exponentially over the last decade. A classification scheme of the bacteriocins of lactic acid bacteria (LAB), a subgroup of Gram-positive bacteria, was proposed by Klaehammer in 1993. Although this scheme was widely accepted by investigators in the field, modifications have been made regarding each original class. Based on the previous revisions of LAB bacteriocin classification schema as well as their laboratory experiences, Heng et al. suggested a new scheme which could apply to not only LAB, but to all bacteriocins of Gram-positive bacteria. Four classes were included (Heng et al., 2007):
- Class I: lanthionine-containing bacteriocins
- Class II: small (<10 kDa), non-modified peptides
- Class III: large (>10 kDa) bacteriocins
- Class IV: cyclic peptides

1.2.1 Class I: Lanthionine-Containing Bacteriocins

Also known as lantibiotics, these bacteriocins contain the unusual thioether amino acids lanthionine (Lan) and/or methyllanthionine (MeLan), as well as other highly modified amino acids, which are not encoded by the conventional 64 triplet genetic code. After being synthesized by ribosomes as a prepeptide, several post-translational modifications take place to produce the biologically active molecule. These events include formation of unsaturated dehydroamino acids by dehydration, addition of sulfhydryl groups to form thioethers, formation and modification of structural elements such as lysinoalanine-bridges, N-terminal blocking groups and in some cases oxidative decarboxylation of the cysteine residue at the C-terminus (Sahl et al., 1995).

Class I lantibiotics can be further divided into three groups based on their structure and number of peptides: type A are elongated amphiphilic peptides having a linear structure, while type B are more compact and globular (Sahl et al., 1995); type C consist of multi (mostly two)-component peptides, which individually have little activity but together act synergistically (Heng et al., 2007).

1.2.1.1 Nisin – an Example of Type A Lantibiotics

Nisin, produced by *Lactococcus lactis*, is the most extensively studied bacteriocin
and is an example of a Class I type A lantibiotic. The biosynthesis of nisin involves more than ten genes, corresponding to a large array of regulatory proteins, including genes whose product(s) show no homology to known genes in the database (van Kraaij et al., 1999). Containing a N-terminal leader sequence, the 57-amino acid nisin precursor is encoded by nisA, which is located on a 70 kb conjugative transposon (Rauch et al., 1991). This precursor peptide is processed by a multimeric protein complex consisting of LanB and LanC proteins which results in the formation of dehydrated residues and eventually the introduction of five lanthionine residues (Peschel et al., 1996). The very last step of precursor processing is the removal of the leader peptide sequence by a subtilisin-like protease, NisP; similar protease-encoding genes were also found in gene clusters for other lantibiotics, such as epidermin and cytolysin (Schnell et al., 1992, Gilmore et al., 1996). It has been suggested that the leader sequence keeps the lantibiotic in an inactive form and protects the producer cell from its activity, but more recent studies of lacticin 481 using point mutations showed that altering the sequence of leader peptide may cause insufficient dehydration modification to amino acids (Patton et al., 2008). The biosynthesis of nisin is regulated by a two-component regulatory system. When nisin concentration reaches a regulatory threshold for the kinase NisK, a signal is initiated by phosphorylation of a specific histidine residue and then transfer to the response regulator NisR, which binds to a corresponding promoter(s) in the nisin gene cluster and turns off transcription (Kuipers et al., 1995).

NisI is responsible for the nisin immunity of producer cells, although the underlying mechanism is not yet fully understood. It is presumed that a sufficient amount of NisI is secreted into the cytoplasm to result in a decrease of the concentration of nisin activity by specific neutralising binding (Koponen et al., 2004). Studies done by Qiao et al. (1995) using nisin-sensitive Lactococcus lactis demonstrated that an additional ABC (ATP-binding cassette) transport protein complex may also
contribute to nisin immunity, by regulating the interaction between nisin and the cytoplasmic membrane (Qiao et al., 1995).

The bacteriocidal activity of type A lantibiotics towards Gram-positive bacteria is much stronger in comparison to that of compounds in the other three bacteriocin classes. The concentration of type A lantibiotics required to kill target cells is a thousand fold less than that of other bacteriocins. The outer cell membrane of Gram-negative bacteria makes them less vulnerable to lantibiotics; however the susceptibility of those cells can be significantly increased upon disruption of the outer cell membrane (Kordel et al., 1986).

The action of nisin on its target cell starts with the binding of the peptide to the cytoplasmic membrane. The C-terminal region of nisin plays a key role in this early interaction, with electrostatic interactions between the positively charged C-terminal residues and negatively charged membrane phospholipids facilitating the interaction of nisin with the cell surface (Breukink et al., 1997). Insertion of nisin into the membrane takes place next and mutation studies using monolayer lipid membranes indicate that the N-terminus is more involved in this process, since mutations of the C-terminal domain had little effect (Demel et al., 1996). Once nisin is inside the bilayer membrane, it orients so that the hydrophobic residues are embedded among the fatty acyl chains, while the hydrophilic residues face towards the membrane-water interface, the subsequent result of this being the formation of multi-peptide pores which allow efflux of low molecular weight components and disruption of the proton motive force, which ultimately leads to cell death (Breukink et al., 1997, Giffard et al., 1997).

Its high antimicrobial efficiency and wide activity spectrum make nisin an attractive candidate for many commercial and pharmaceutical applications. In industrial cheese
production, nisin-producing strains of *L. lactis* have been added to the fermentation microflora to prevent the over growth of spores by antagonizing the cheese-spoilage bacterium *Clostridium tyrobutyricum* (Vandenbergh, 1993). From a pharmaceutical perspective, recent studies with rabbits suggested a potential application of nisin as a contraceptive. Vaginal administration of 1 mg of nisin in female rabbits prevented them from achieving pregnancy by eliminating sperm motility (Reddy *et al.*, 2004). Rational design of new nisin variants with improvements in nisin solubility at physiological pH may broaden the medical applications of nisin, so that it can be used intravenously (Rollema *et al.*, 1995).

1.2.1.2 Type B and Type C lantibiotics

The fundamental elements of nisin biosynthesis are also conserved with the type B and type C lantibiotics. Minor variations include structural differences in the unsaturated and/or Lan residue modifications and formation of additional structural elements such as the ring structure of type B lantibiotics. Mersacidin, produced by *Bacillus* sp. strain HIL Y-85, 54728, is a type B lantibiotic with three distinctive MeLan rings, and a more compact size of 20 amino acids (compared to 34 in nisin) (Chatterjee *et al.*, 1992). Unlike the type A lantibiotics, mersacidin does not form pores in the cytoplasmic membrane, but instead it inhibits cell wall synthesis by targeting the cell wall precursor – lipid II (Brötz *et al.*, 1995). NMR studies of mersacidin-lipid II interactions revealed that conformational change plays a key role in the inhibitory activity, and like the interaction between nisin and the cell bilayer membrane, electrostatic charge is also involved in lipid II interactions (Hsu *et al.*, 2003). Mersacidin is well-known for its potent activity against methicillin-resistant variants of *Staphylococcus aureus*. Unlike vancomycin, which has also been widely used to treat serious methicillin-resistant *S. aureus* infections, the inhibitory activity of mersacidin to cell wall synthesis is not antagonized by diacyl-tripeptide (Brötz *et
Lacticin 3147, produced by *Lactococcus lactis* subsp. *Lactis* 3147, is a pore-forming bacteriocin consisting of two peptides: A1 and A2, working synergistically in a sequential pathway. Structural analysis using multidimensional-NMR spectrometry has indicated that the peptide A1 has a similar structure to the globular type B lantibiotics, while peptide A2 adopts an elongated linear conformation like that of type A lantibiotics (Martin *et al*., 2004). During the action of lacticin 3147, an independent low-level inhibitory activity is initiated by the binding of peptide A1 to lipid II, which is followed by the interaction of peptide A2 with the A1-lipid II complex to achieve a more effective membrane insertion and pore formation. The participation of peptide A2 results in an increase of the inhibitory activity by more than 30-fold (Morgan *et al*., 2005).

1.3 Class II and Class IV bacteriocins

Class II bacteriocins include the non-lantibiotic and non-cyclic, low molecular weight (< 10 kDa) peptides from Gram-positive bacteria. This class comprises more than 50 members of functionally diverse bacteriocins from different producer species and strains, derived from either human or other animal origins. The activities of Class II bacteriocins are either introduced by their own intrinsic single or multi-component bioactive peptides, or by extrinsically stimulating the biosynthesis of another inhibitory substance, usually another bacteriocin (Eijsink *et al*., 2002).

Pediocin-like antimicrobial peptides, consisting of more than 20 members, are typical representatives of the single functional peptide type of Class II bacteriocins. The N-terminus of these pediocin-like AMPs (antimicrobial peptides) contains a conserved YGNGV amino acid sequence motif and two or four (depending on which
variant) cysteine residues forming disulfide bridge(s). Both structural elements are involved in the antimicrobial activity (Eijsink et al., 1998). The C-terminal residues of the pediocin-like bacteriocins are less conserved and studies have showed that variations of the amino acid composition in the C-terminal region may play a role in their inhibitory spectrum determination (Fimland et al., 1996). The mode of action of pediocin-like bacteriocins is similar to that of type A lantibiotics and comprises the three conventional steps of binding, insertion and poration of the cytoplasmic membrane. Penetration of pediocin-like AMPs into the membrane is facilitated by the hinge motif between the N-terminal $\beta$-sheet region and the C-terminal hairpin region (Fimland et al., 2005).

In comparison to the Class I and Class II bacteriocins mentioned above, the Class IV bacteriocins are less common and less well defined. Class IV comprises the post-translationally modified cyclic bacteriocins whose first amino acid and last amino acid are covalently joined, featuring a head-to-tail linkage (Maqueda et al., 2004).

Enterocin AS-48 is produced by Enterococcus faecalis subsp. liquefaciens. Unlike other lantibiotics, no thioether residues are present in the structure of enterocin AS-48. Instead, a unique peptide link was established between the C-terminus Trp-70 and N-terminus Met-1 (Maqueda et al., 2004). A remarkable characteristic of enterocin AS-48 is its resistance to heat and stability over a wide pH range, which makes it an ideal candidate as a food preservative. Indeed, a study using enterocin AS-48 as a preservative in canned food has validated its effectiveness at inhibiting undesired bacterial species, such as Bacillus coagulans. Enterocin AS-48 also has enhanced anti-spore activity when combined with high temperature thermal treatment (Lucas et al., 2006). Discovery of a novel cyclic bacteriocin was recently reported by Japanese scientists. Termed lactocyclicin Q, it is the very first cyclic
peptide purified from *Lactococcus* species and is a new potential producer class for cyclic bacteriocins (Sawa *et al*., 2009).

### 1.4 Class III Bacteriocins and Salivaricin MPS

The Class III bacteriocins are larger (> 10 kDa), heat labile antimicrobial molecules, mostly produced by streptococcal bacteria. Zoocin A is produced by *Streptococcus equi sp. zooepidemicus*, and is a 27.8 kDa, 262-aa macromolecule with structural and functional homology to lysostaphin, the prototype staphylococcal bacteriolysin (Heng *et al*., 2007, Simmonds *et al*., 1997). The C-terminal domain of zoocin A is involved in substrate binding with a putative receptor-recognition region, while the N-terminal domain is responsible for the peptidase activity of the molecule, which targets the interpeptide crossbridge to affect cell wall hydrolysis.

Among the handful of members of the Class III bacteriocins, dysgalacticin is a typical representative of the bacteriocins whose mode of action involves a non-lytic pathway. Unlike the lantibiotics, dysgalacticin displays a rather narrow spectrum for its inhibitory activity. In contrast to zoocin A, dysgalacticin does not lyse its target cell, but instead it inhibits sugar uptake into the target cell by blocking the phosphotransferase system, which eventually leads to loss of intracellular potassium ions and disruption of membrane integrity, as well as ATP starvation (Swe *et al*., 2009).

Salivaricin MPS, produced by *S. salivarius* strain MPS, has shown several common features consistent with the definition of a Class III bacteriocin. Salivaricin MPS is a 575 aa macromolecule with a mass of 62 kDa, which surpasses the size of most (if not all) of the current known bacteriocins of LAB. It is a heat-labile bacteriocin and loses biological activity above 50°C (Dodd, 1999).
PCR of *S. salivarius* strain MPS using a salivaricin MPS DNA leader sequence primer identified a secondary variant gene – *salMPS*var, distinct from the original *salMPS* gene. Both genes appear to generate translated products. *In silico* analysis of the putative translated products of the two genes showed a high amino acid sequence composition similarity and almost identical theoretical pI for the two proteins. BLAST searches against available bacterial protein databases returned no homologies with any known microbial proteins (Wang, 2007).

In addition to the fact that no match was found with any other proteins, the producer strain *S. salivarius* MPS also displays several unique properties which attracted attention. The production of salivaricin MPS-related anti-microbial activity takes place at an early stage of growth of producer strain cultures under laboratory growth conditions, typically starting between four to five hours after inoculation of liquid cultures (Dodd, 1999). By contrast, the detection of the inhibitory activities of most other bacteriocins by *S. salivarius* does not occur until the cultures have entered later logarithmic to early stationary phase. The inhibitory spectrum of strain MPS covers a wide range of Gram positive bacteria, including strains of *Micrococcus* spp. and *Lactococcus* spp. Of particular interest, is the strong activity of salMPS against *S. pyogenes*, a common pathogen of humans. *S. salivarius* strain MPS, exhibits a production type (P-type) of 636 in standard deferred antagonism tests (Tagg and Bannister, 1979), a pattern of inhibition that indicates the ability of salMPS to inhibit all four *S. pyogenes* indicator strains within the set of nine standard indicators. Furthermore, since *S. pyogenes*, *S. salivarius* is (like *S. pyogenes*) also commonly found in the human oral cavity, strain MPS can be considered to have excellent potential for application as probiotic for clinical bacterial interference purposes.
The aim of this present study was to purify and characterize the salMPS-like activity from selected *S. pyogenes*-specific producers, in particular from *Streptococcus uberis* NY42, which may have the potential to produce the prototype salMPS; to compare and contrast this activity with that produced by *S. salivarius* strain MPS, and to contribute to knowledge of this novel macromolecule regarding its production and mode of action by different streptococcal species.
2.1 Salivaricin MPS Producer Strains and Indicator Strains

The previously-studied salivaricin MPS producer strains *Streptococcus salivarius* strains MPS and Mia were provided by Prof. John Tagg of the Department of Microbiology and Immunology, University of Otago.

The nine standard indicator strains routinely used in a deferred antagonism test for P-typing of BLIS (bacteriocin like inhibitory substance) -producing bacterial strains were also obtained from Prof. Tagg. The species and strain names of the nine standard indicators are listed in Table 2.1.1. These strains and also *Streptococcus pyogenes* strain EB1, which was used later in this project as an additional sensitive indicator strain, were also provided by Prof. Tagg. Strain EB1 was previously reported to be especially sensitive to salivaricin MPS (Dodd, 1999).

The producer and indicator strains were sub-cultured on BACa (calcium carbonate supplemented blood agar) medium, with incubation for 18 h at 37°C in a 5% CO₂ in air atmosphere. These cultures were stored at 4°C until required for use. All strains were sub-cultured weekly.
Table 2.1.1 Standard Indicators

<table>
<thead>
<tr>
<th>Standard Indicator</th>
<th>Species</th>
<th>Strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>I₁</td>
<td><em>Micrococcus luteus</em></td>
<td>T-18</td>
</tr>
<tr>
<td>I₂</td>
<td><em>Streptococcus pyogenes</em></td>
<td>FF22</td>
</tr>
<tr>
<td>I₃</td>
<td><em>Streptococcus anginosus</em></td>
<td>T-29</td>
</tr>
<tr>
<td>I₄</td>
<td><em>Streptococcus uberis</em></td>
<td>ATCC27958</td>
</tr>
<tr>
<td>I₅</td>
<td><em>Streptococcus pyogenes</em></td>
<td>71-679</td>
</tr>
<tr>
<td>I₆</td>
<td><em>Lactococcus lactis</em></td>
<td>T-21</td>
</tr>
<tr>
<td>I₇</td>
<td><em>Streptococcus pyogenes</em></td>
<td>71-698</td>
</tr>
<tr>
<td>I₈</td>
<td><em>Streptococcus pyogenes</em></td>
<td>W-1</td>
</tr>
<tr>
<td>I₉</td>
<td><em>Streptococcus equisimilis</em></td>
<td>T-148</td>
</tr>
</tbody>
</table>

2.2 Bacterial growth Media and Cultures

Todd Hewitt Broth (THB) and the routinely-used BACa, were prepared from Bacto Todd Hewitt Broth Base and Difco Columbia Blood Agar Base (respectively) according to the manufacturer’s instructions. Supplements of 0.1% (w/v) calcium carbonate and 5% (v/v) whole human blood were included in the BACa medium. CAB (Columbia agar base) medium preparation was similar to that of BACa, but without addition of calcium carbonate and human blood.

A variation to the standard THB above was the preparation of dialysate THB (dTHB), which was prepared by dialysis of the regular THB. An appropriate length of 8,000 Da MWCO (molecular weight cut-off) dialysis membrane tubing was soaked in distilled water until the tubing could be opened easily. One volume of 10 x concentrated regular THB was transferred into the dialysis tubing and both ends were sealed with clips and the preparation was dialyzed against 9 volumes of distilled water overnight at 4°C. The THB dialysate (outside the dialysis tubing) was then dispensed into smaller volumes before autoclaving and storage at 4°C.
2.3 Sterilization and Storage Conditions

2.3.1 Sterilization and storage of growth media

Liquid media, such as THB used in this project, were steam autoclaved at 121°C for 15 min and cooled to room temperature prior to use.

The preparations of agar media were autoclaved under the same conditions used for liquid media and cooled to 55°C in a water bath before pouring to form agar plates in petri dishes. After the agar had set, the plates were inverted overnight to avoid condensate from forming on the lids and dropping onto the agar surface.

Both the liquid and solid agar media were stored at 4 °C.

2.3.2 Chloroform Sterilization

The agar surface to be sterilized was inverted over a chloroform-soaked cloth pad on a glass surface for 30 min, followed by airing of the agar surface for another 30 min at room temperature to allow residual chloroform to evaporate.

2.3.3 UV Irradiation Sterilization

The agar surface to be sterilized was exposed directly (~40 cm distance) under a UV light source (wavelength 270 nm) for at least 40 min in a covered hood at room temperature.
2.4 Salivaricin MPS Production from *S. salivarius* Strain MPS

2.4.1 Salivaricin MPS Production in Modified Liquid Medium

The method used was that reported previously for liquid medium inhibitor production (Dodd, 1999). One mL of an 18 h THB culture (37°C, 5% CO₂) of *S. salivarius* MPS was inoculated into 16 mL of sterilized THB supplemented with 1 mL of 10% (w/v) glucose and 2 mL of 0.5 M CaCO₃, (final concentrations of glucose and CaCO₃ of 0.5% and 0.05 M, respectively). Cysteine was also added to a final concentration of 5 µg.mL⁻¹. Incubation was at 37°C and samples were taken for testing at 3 h and 5 h (short term production). The culture samples were centrifuged at 16,100 g for 5 min and the supernatants assayed on CAB using the well diffusion (see below) method for inhibitory activity against selected indicators.

2.4.2 Salivaricin MPS Production in bi-phasic medium

A lawn of the producer strain was seeded onto the surface of BACa and grown for 18 h (37 °C, 5% CO₂) before addition of 5 mL of THB to the surface of the BACa. The plate was then returned to the incubator, taking care not to spill the top (liquid) layer, and 500 µL samples were taken of this fluid every 2 h (from 3 h to 9 h). Following centrifugation (16,100 g, 5 min) the supernatant fluid was assayed against selected indicators by the well-diffusion method (see 2.5.3 for more details).
2.5 Tests for Inhibitory Activity

Unless otherwise stated, all of the indicator assays described below incorporated aerobic incubation at 37°C, with 5% CO₂ in the atmosphere.

2.5.1 Simultaneous Antagonism Test

Indicator strains were swabbed on the surface of BACa agar plates using sterile cotton swabs. A sterile toothpick was then used to collect a sample of the bacterium to be tested by sampling from a representative colony (or closely-similar colonies) on the surface of a fresh agar culture of the bacterium and then stabbing into the lawn newly-seeded on BACa. The assay plate(s) were then incubated for 18 h at 37°C. Inhibitory activity of the producer strain against the indicator strain was manifest by a clear zone of no indicator growth around the site where the test bacterium had been stabbed into the agar.

2.5.2 Deferred Antagonism Test

The putative producer strain was streaked diametrically across a BACa agar plate as a 1 cm wide strip, using a sterile cotton swab. The assay plates were incubated for 18 h under the appropriate conditions. THB cultures (3 mL) of the indicator strains were incubated aerobically at 37°C for 18 h. The visible growth of the bacterial streak culture was then scraped off using the edge of a glass microscope slide, followed by chloroform or UV sterilization of the agar surface. The indicator strains were then streaked from one side of the plate to the other, perpendicular to the centre producer growth region, and the assay plate was re-incubated aerobically for 18 h, at 37°C. The production type (P-type) of the test strain was determined from the inhibitory pattern given against the nine standard indicators (I₁ – I₉).
2.5.3 Well Diffusion and Spot Diffusion Assays

After centrifugation (16,100 g, 5 min) 50 µL of supernatant was loaded into wells (diameter ~0.5 cm) that had been cut into the CAB assay agar using the reverse end of a sterile glass pipette. The bases of these sample wells had been sealed with 20 µL drops of Davis agar. The samples were allowed to diffuse into the agar for at least 40 min at 4°C. The assay plates were then chloroform sterilized. A 1:100 dilution (in distilled water) of an 18 h THB culture of the indicator strain was then gently and evenly swabbed onto the surface of the assay medium. The assay plates were then incubated for 18 h, at 37°C.

In the heat stability test, a heating step was included prior to sample loading. The test samples (in 1 mL microfuge tubes) were held in an 80°C water bath for 30 min. The heated samples were then centrifuged at 16,000 g for 2 min (to pellet denatured protein aggregate) before aliquots of the supernatants were loaded into wells (cut into CAB agar as above).

The method for spot diffusion assays was similar to that of the well diffusion assay, except that 25 µL test samples were directly applied to the surface of the agar assay plate, and held at 4°C until all of the liquid had been absorbed into the agar.

2.6 Polymerase Chain Reaction (PCR) Screening for Potential Producers of salMPS-like Activity

The test strains were first assayed for their P-type using deferred antagonism against the nine standard indicators. Strains whose P-type pattern included strong activity against indicators I_2, I_5 and I_7 (these three S. pyogenes are most sensitive to the bacteriocin salMPS) were selected for PCR-screening using the primer pairs designed to amplify the original salMPS (salMPS) and the variant salMPS (salMPSvar) genes.
Primers for PCR were designed based on the DNA sequence obtained by Dodd (Dodd, 1999), to differentially amplify \textit{salMPS} and the \textit{salMPSvar}. The primers for the two genes were:

- TATTTCGATGATGGCGGGTATGGA \textit{salMPS} forward primer
- CGTGGCCGAATGCTGCAATACCTTA \textit{salMPSvar} forward primer
- ACTTTCCGTATTATTTGTTGGTTCG reverse primer

The same reverse primer was used for both \textit{salMPS} and \textit{salMPSvar}. A negative control PCR reaction containing no DNA was prepared and separately-prepared DNA samples of \textit{S. salivarius} strains Mia and MPS were used as the positive controls.

\textbf{2.7 Extraction of prototype salMPS inhibitor from BACa agar cultures}

\textbf{2.7.1 Freeze-thaw extraction and urea extraction of salMPS-like inhibitory agents}

An 18 h THB culture of the producer strain was evenly swabbed onto BACa agar plates and these were incubated aerobically with 5\% CO$_2$ for 21 h before transferring to a -80\(^\circ\)C freezer for 4 h. The frozen plates were then inverted at an angle above the petri dish lids for collection of the exudate fluid draining from the frozen cultures as they thawed at room temperature. The exudate was transferred to universal containers and stored at 4\(^\circ\)C. Typically, 75 BACa cultures produced approximately 100 mL of exudate.

For urea extraction, 20 BACa agar plates were seeded with the producer strain and incubated at 37\(^\circ\)C for 21 h. The culture lawns were then removed from the agar surface using a sterile microscope slide and re-suspended in 40 mL 7 M urea. The urea suspension was then kept on ice for 3 h to allow for extraction of the inhibitor, and then the solution was transferred to a 50 mL Falcon tube and centrifuged at 2,000
g for 5 min. The supernatant was collected and repeatedly re-centrifuged until no visible cell pellet remained in suspension. The clear supernatant and the cell pellets were stored at 4°C for further experiments.

2.7.2 Attempted removal of haemoglobin from extractions using urea and guanidine hydrochloride (GuHCl)

A drawback of using BACa agar as a growth medium for freeze/thaw extraction was the presence of a large amount of haemoglobin in the exudate. Since the haemoglobin tetramer has a molecular weight of 68 kDa, which is close to the 62 kDa salMPS molecule, it presented a challenge for subsequent FPLC fractionation, in that the haemoglobin co-eluted with the inhibitory activity on ion exchange chromatography. To overcome this potential problem, attempts were made to remove the haemoglobin using two denaturing agents: urea and GuHCl, with the hope that by disrupting the tetrameric structure of haemoglobin, it could be separated from the inhibitory activity based on size difference.

To minimise the effect of volume increase on the final concentration of urea, 2.4 g of urea was added to 5 mL of strain NY42 BACa freeze thaw extract to achieve a final concentration of 8 M urea. The mixture was inverted gently until all of the urea was dissolved. A 200 µL sample of this preparation was kept at 4°C for inhibitory activity assay, and the rest transferred to a 30 kDa MWCO Vivaspin centrifuge filter unit (GE Healthcare) and centrifuged at 9,000 g for 15 min at 4°C. Both the filtrate and retentate were assayed against S. pyogenes indicators I7 and EB1, along with an aliquot of the 200 µL sample as the control.

GuHCl solutions ranging from 1 M to 6 M were prepared (1 mL of each) in 1.5 mL microfuge tubes. To each tube 200 µL of strain NY42 BACa freeze thaw exudate was added and mixed gently by inverting. The mixture was kept at 4°C for 1 h and then spot assayed against I7 and EB1 to test for retention of inhibitory activity in presence
of GuHCl. GuHCl solutions without addition of the freeze thaw extract were also assayed, as was a sample containing 200 µL freeze thaw extract as a positive control.

2.7.3 Ammonium Sulfate precipitation

Crude freeze-thaw extract (165 mL) of *S. uberis* strain NY42 from BACa agar was stored at 4°C. A 3 mL aliquot was kept for later assays. To the remainder, ammonium sulfate was added to attain 50% saturation. After 40 min standing, the ammonium sulfate treated preparation (crude NY42) was centrifuged at 10,000 g for 20 min, at 4°C. The precipitate was dissolved in a minimal volume of phosphate buffered saline (PBS) pH 7.0, and the supernatant was stored at 4°C after centrifugation to remove any non-soluble material.

This preparation (crude NY42) was transferred to 8,000 Da cut-off dialysis membrane tubing and dialysed against 1 L of pH 7.0 PBS overnight at 4°C. The dialyzed sample was centrifuged at 16,100 g for 5 min and the supernatant stored at 4°C (dialysed crude NY42).

2.7.4 Adsorption to and elution of inhibitory agents from viable or from heat-killed *S. pyogenes* cells

The cells from BACa lawn cultures of *S. pyogenes* EB1 and indicator I_7_ were used in studies to assess binding of the prototype salMPS inhibitors. A 1 x 18 h lawn culture (BACa) of EB1 and I_6_ were scraped from the agar surface using a sterile glass slide and re-suspended in 3 mL of pH 7.0 PBS. Aliquots of the cell suspensions were either heated at 80°C for 30 min to kill the cells or kept at room temperature (as viable cells). After centrifuging at 16,100 g for 5 min, the supernatant was carefully removed without disturbing the cell pellet. Two mL of *S. uberis* NY42 freeze-thaw BACa extract was gently mixed with the cell pellet and incubated for 40 min at 4°C with mixing by swirling every 10 min.
The supernatants of these incubated preparations were transferred to 1.5 mL microfuge tubes and kept on ice, after centrifugation at 16,100 g for 5 min. Serial 1:2 dilutions (in distilled water) of the supernatants were tested by well-diffusion assay for inhibitory activity. The reciprocal of the highest dilution which still produced a visible inhibitory zone was recorded as the endpoint titre for that particular sample.

Either NaCl (1 M or 2 M), or 7 M urea was added to microfuge tubes containing the \textit{S. pyogenes} or \textit{L. lactis} cells and briefly mixed by tapping the bottom of the tube, followed by centrifuging at 16,100 g for 2 min. The supernatant samples were then well assayed on CAB agar for inhibitory activity. Dilutions (1:1 with NaCl or urea) of the NY42 BACa freeze thaw extract samples were prepared and used as the control for the activity assay.

\textbf{2.8 Gel permeation HPLC of dialysed crude NY42 preparations}

Prior to loading samples onto a gel permeation chromatography column (Superdex 200 10/300 GL), the HPLC system (Applied Biosystems, 140A Solvent Delivery System) and column was equilibrated in 20 mM HEPES, pH 7.2, containing 150 mM NaCl.

An aliquot (600 µL) of the dialysed crude NY42 preparation was clarified by centrifugation at 16,100 g for 5 min and 0.5 mL of the supernatant injected onto the HPLC column at a flow rate of 0.5 mL.min$^{-1}$. Fractions (1 min, 0.5 mL) were collected between 15 and 45 min after injection, with detection at 280 nm (Applied Biosystems, 1000S Diode Array Detector).

All collected fractions were held on ice until being spot tested for inhibitory activity against \textit{S. pyogenes} EB1 and standard indicator I$_7$. The non-fractionated dialysed crude NY42 preparation was used as the control. A back mix consisting of a
combination of a 20 µL aliquot from each of the peak fractions F5, F6, F7, F12, F18 and F27 was also assayed for inhibitory activity. Fractions displaying inhibitory activity against *S. pyogenes* were also assayed for activity against indicators I₁ and I₆.

2.8.1 SDS-PAGE of inhibitory fractions from the dialysed crude NY42 preparation

Aliquot (400 µL) of each of the two active fractions (F18 and F19) and four inactive fractions (F14, F16, F21 and F24) from the first run of the HPLC were transferred to six individual 30 kDa MWCO centrifuge filter tubes (GE Healthcare, Vivaspin 500), and centrifuged at 10,000 *g* for 1 h, at 4°C (Beckman Coulter, Allegra X-15R Centrifuge).

An aliquot (15 µL) of each of the above concentrated fraction preparations were mixed with 5 µL of NuPAGE sample buffer (Invitrogen), 1 µL dithiothreitol (4.8%, v/v) and briefly warmed at 70°C for 5 min, before loading on a NuPAGE 4-12% gel (Invitrogen). The upper and lower tank chambers were both filled with 1 x NuPAGE MES SDS electrode buffer (with 500 µL of antioxidant added to the upper chamber) and the gel was electrophoresed at 115 V, 35 mA, for 1 h.

The gel was rinsed with sterile MilliQ water twice for 5 min before being stained with 20 mL of SimplyBlue SafeStain (Invitrogen) for 1 hr, and subsequently destained with 2 % (v/v) NaCl in water overnight. Images of the gels were captured using a Canon scanner.

Regions of the stained SDS-PAGE gel between 30 and 80 kDa were excised from the gel and also a significant low molecular weight band (~16 kDa) were each digested with 100 µg trypsin and the peptides extracted from the gel prior to analysis using MALDI TOF MS/MS and LC-Orbitrap HPLC MS by the Centre for Protein Research, University of Otago. The MS results were searched online using the SwissProt database for a broad-base search of protein homologies among all available sequences.
from different species, and with the NCBI Streptococcus database for a more specific search of possible sequence matches within the streptococcus genus. The translated peptide sequences of salMPS and salMPSvar were also provided as user defined sequence library for further sequence homology searches.

2.9 Ion-Exchange Chromatography

Table 2.9.1 Ion-exchange columns and buffers

<table>
<thead>
<tr>
<th>Column*</th>
<th>Buffer A</th>
<th>Buffer B</th>
</tr>
</thead>
<tbody>
<tr>
<td>CM FF (1 mL)</td>
<td>20 mM MES, pH 5.8</td>
<td>20 mM Mes, 2 M NaCl, pH 5.8</td>
</tr>
<tr>
<td>DEAE FF (1 mL)</td>
<td>20 mM Tris, pH 8.0</td>
<td>20 mM Tris, 2 M NaCl, pH 8.0</td>
</tr>
</tbody>
</table>

* CM FF – carboxymethyl Sepharose, fast flow (weak cation exchanger); DEAE FF – diethylaminoethyl Sepharose, fast flow (weak anion exchanger). Both ion-exchange columns had a 1 mL packed bed volume and were stored at 4°C, in 20% (v/v) ethanol.

One volume of 80% ammonium sulfate-precipitated freeze thaw extract of *S. uberis* strain NY42 from BACa agar was mixed with 4 volumes of MilliQ water and re-concentrated to 1 volume using a 30 kDa MWCO Vivaspin spin concentrator filter (9000 g, 4°C); the process was repeated three times and the buffer exchanged material was assayed for inhibitory activity against *S. pyogenes* EB1.

Desalted NY42 freeze thaw extract (10 mL) was manually injected onto the column (DEAE or CM previously equilibrated in respective Buffer A) at a flow rate of 1 mL.min⁻¹. The column effluent was collected and assayed against *S. pyogenes* EB1. The column was then washed with 10 mL of buffer A (Table 2.9.1) and the effluent collected was designated as the “unbound” fraction. Six fractions were collected during the buffer B wash and were assayed against indicators EB1 for inhibitory
activity.

An aliquot (2 mL) of the flow-through material (buffer exchanged with MilliQ water) from the preliminary CM column binding experiment was loaded onto the DEAE column which was connected to an ÄKTAprime plus FPLC system for anion-exchange chromatography. One mL fractions (flow rate: 1 mL.min$^{-1}$) were collected with UV detection at 280 nm. All fractions were subsequently assayed for inhibitory activity against indicators $S$. pyogenes EB1 and I1.

To further investigate whether the CM ion-exchange column could be a useful procedure to help remove the haemoglobin content from NY42 freeze thaw extract, 2 x 1.5 mL of CM Sepharose (fast flow, Pharmacia) was transferred to two 2 mL microfuge tubes and washed in 20 mM Tris buffer (pH 8.0), the resin was compacted and the supernatant was removed after centrifugation at 11,000 g for 3 min, and this process was repeated twice. Approximate equal volumes of MilliQ water-exchanged, ammonium sulfate precipitated (80% saturation) NY42 freeze thaw extract was added to one (tube 1) of the two CM Sepharose tubes and mixed gently by inverting the tube, during the incubation at room temperature for 20 min to allow binding of haemoglobin to the CM resin. An aliquot of the supernatant (tube 1) was kept for inhibitory activity assay after centrifugation and the rest of the supernatant was transferred to the second CM Sepharose tube (tube 2) for further haemoglobin binding. The supernatant was removed after centrifugation and assayed for inhibitory activity against $S$. pyogenes EB1.
Chapter 3

Results and Discussion

3.1 Salivaricin MPS production by *Streptococcus salivarius* strains MPS

3.1.1 Salivaricin MPS production using solid growth media

Production typing (P-type) of salivaricin MPS-producing *S. salivarius* strains

P-typing is a system developed by Tagg and Bannister (1979), in which the P-type is represented by a three digit number, with each digit corresponding to the total score of the inhibited standard indicators from one of the three groups (each group consisting of 3 indicators in numerical order, e.g. group one includes I₁, I₂ and I₃, group two includes I₄, I₅ and I₆, and the rest for group three). The production types of *S. salivarius* strain MPS and strain Mia, which were previously used as typical producers of salivaricin MPS, are shown in Table 3.1.1.1.

Table 3.1.1.1 P-types of salivaricin MPS-producing *S. salivarius* strains MPS and Mia under aerobic and anaerobic growth conditions

<table>
<thead>
<tr>
<th>Producer</th>
<th>Degree of inhibition of indicator</th>
<th>P-type</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I₁</td>
<td>I₂</td>
</tr>
<tr>
<td>MPS</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>MPS&lt;sub&gt;An&lt;/sub&gt;</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Mia</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>Mia&lt;sub&gt;An&lt;/sub&gt;</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

The nine standard indicators (I₁ to I₉, see Materials and Methods, Table 2.1.1) were divided into three groups, with each group consisting of three indicators. The first
indicator of each group scores 4, the second one scores 2 and the last one scores 1. Using MPS as an example, under aerobic conditions, the first two indicators of group one were inhibited, which gave a total score of 6 (4+2). Using the same algorithm, the total score of group two and group three were calculated to be 3 and 6 respectively. The final P-type was 636, with each digit reflecting the total score of inhibited indicators from each group of the nine indicators.

The superscript “An” indicates that the producer strain was grown under anaerobic conditions, with the temperature (37°C) and the time (18 h) of incubation being the same as for aerobic growth conditions.

The width of inhibitory zone against a particular indicator is indicated by the number of “+” marks, one “+” mark represents the width of the zone being equal or within the producer growth streak (~1 cm wide), two “+” corresponds to an inhibitory zone width that is up to twice the width of the producer streak; three “+” is where the indicator growth is inhibited beyond twice the width of the producer streak zone.

The superscript “R” above the “+” indicates that there were single resistant colonies or some visible colony growth within the inhibition zone.

The P-types of *S. salivarius* MPS (636) and of Mia (677) included a consistent anti-*S. pyogenes* component (i.e. 226 P-type activity), as expected from the previous studies by Dodd (Dodd, 1999) and myself (Wang, 2007). All four *S. pyogenes* indicators (I₂, I₅, I₇ and I₉) were inhibited by both producer strains under aerobic (CO₂ supplemented) growth conditions. The overall activity of strain Mia was stronger than that of strain MPS, as indicated by the wider inhibitory zone sizes on all sensitive indicators, as well as the additional inhibitory activity of strain Mia on I₄ and I₉. This was not surprising, as strain Mia has previously been shown to have the potential to produce more than one inhibitor, indicated by the heat stability assay, which showed strain Mia was capable of producing both small heat-stable inhibitor(s) and large heat-sensitive
inhibitor(s) (Wang, 2007). The stronger inhibitory activity of strain Mia may be due to the activity of additional inhibitors or to the combined effect of several inhibitors. Both producers showed a reduction in their activity when grown anaerobically, with activity on I₈ and I₅ being lost and resulting in a P-type for MPS of 634 and for Mia of 657. The zone sizes on the other sensitive indicators were decreased, and resistant colonies of the indicators were more likely to appear. One possible reason could be that the amount of inhibitory agent released by the producers under anaerobic conditions was insufficient to kill all of the indicator cells within the inhibitory zone.

Simultaneous antagonism testing of *S. salivarius* strains MPS and Mia

Both strain MPS and strain Mia were inhibitory to indicators I₁, I₂, and I₅, but not to indicators I₇ and I₈ in this test. The inhibitory zones produced in simultaneous antagonism were larger for strain Mia than for strain MPS. This may be due to the production of additional inhibitory substances by strain Mia. This difference was most obvious against indicator I₁, which had an inhibitory zone size of 1.2 cm radius for strain Mia compared to 0.5 cm for MPS. This may be due to different amounts of inhibitor(s) produced by strains Mia and MPS or to different specific activities of the different inhibitors. The diameter of the inhibitory zones against indicators I₂ and I₅ were much smaller (0.6 cm and 0.5 cm for Mia and 0.3 and 0.2 cm for MPS) for both producer strains, in which case the zone sizes from strains MPS were just slightly larger than the colony size around the stabbing point. However, the edges of these zones were still very well defined. In addition, a wider region surrounding the inhibitory zones of strain MPS were observed against I₅, as indicated in Figure 3.1.1.2 (b). These zones may represent the effect of an enzyme released by strain MPS on capsule formation by the *S. pyogenes* indicator strain.
Figure 3.1.1.2 P-typing and simultaneous antagonism tests of *S. salivarius* strains Mia and MPS

(a) Left plate: *S. salivarius* strain Mia displays a 677 P-type pattern. Right plate: *S. salivarius* strain MPS is P-type 636 in this example. Indicators from top to bottom – *S. pyogenes* EBI; standard indicators 1 – 9 (see Table 2.2.1 for more details).

(b) Simultaneous antagonism testing of duplicate applications of strains Mia (top) and MPS (bottom) on indicators I₁ (left plate) and I₅ (right plate); black arrows (MPS, right plate) indicate the edge of a faint circular region of altered growth of the indicator lawn.
The diminished *S. pyogenes*-specific inhibitory activity observed from the simultaneous antagonism test was possibly affected by the difference between the concentration of the inhibitors released and the target cells. It is likely that in the deferred antagonism test, the broad producer streak resulted in a relatively high concentration of inhibitor or inhibitors prior to indicator growth. Whereas in the simultaneous antagonism test, a smaller number of inhibitor molecules were likely to have been produced by only a few colonies stabbed onto the agar, and as the producer and the indicator strains grow at the same time, this may also limit the diffusion of the released inhibitor molecules to a shorter distance within the assaying agar medium, thus resulting in smaller inhibitory zones.

The faint circular regions of altered growth of the I$_2$ and I$_5$ lawns around the stab cultures of strain MPS may also be explained by the above rationale, as fewer inhibitor molecules may have diffused across the agar medium from the production source, and the local concentration of the inhibitor may have been insufficient to kill enough target cells to give a clear zone, resulting in a faint region with a lighter growth of the indicator lawn.

The P-type of both *S. salivarius* MPS and Mia matched the results of the previous study under aerobic conditions, although the simultaneous assay showed variation in terms of the pyogenes-specific spectrum and activity strength. However the anti-I$_2$ and anti-I$_5$ inhibitory activities did appear to be consistent.

3.1.2 Salivaricin MPS production in a modified liquid growth medium

No anti-*S. pyogenes* activity was detected in supernatant samples obtained from cultures of strain MPS in either dialysate THB or dialysate THB supplemented with glucose, CaCO$_3$ and Cys. Neither was any activity detected in cultures grown in regular THB medium. Similar experiences had been encountered in the previous salivaricin MPS production studies, whenever THB was used as a liquid growth
medium. As strain MPS did not produce any detectable activities in any of the three liquid culture media, this not only challenges the hypothesis that salMPS was responsible for the fast onset of anti-pyogenes activity during the early stage of the *S. salivarius* producer strain growth, but also contradicted the idea proposed by Dodd (1999) that by supplementing the THB with cysteine and glucose, the production of the anti-*S. pyogenes* activity by *S. salivarius* MPS could be enhanced.

It was a surprise that the results showed no activity of *S. salivarius* MPS against I₁, the most sensitive indicator to most *S. salivarius* BLIS producers in antagonism tests. In addition, no inhibitory activity was detected when the producer was grown in liquid cultures. The loss of salMPS activity in liquid cultures of strain MPS was also reported by Steve Dodd in his project. The results of Dodd indicated that anti-I₁ activity was not detectable in either 5 h CDM (chemically defined medium) or THB cultures of *S. salivarius* MPS when re-assayed after 8 h storage at 4°C. However, inhibitory activity could be restored by addition of cysteine (1% w/v). In the present study, the short term production result suggested that the loss of anti-pyogenes activity of strain MPS in THB medium was irreversible, since addition of cysteine did not restore inhibitory activity.

Unlike strain MPS, the anti-I₁ activity of strain Mia was detected in 3 h and 5 h samples. A slight increase in strain Mia anti-I₁ inhibitory activity was detected in dialysate THB, but the use of dialysate THB medium did not reproduce the anti-*S. pyogenes* inhibitory activity of strain Mia. This indicated that the activity spectrum observed for strain Mia in the antagonism test is due to the combined action of more than one inhibitory molecule, since the anti-I₁ activity was produced independently of the anti-*S. pyogenes* activity.

Purity checks of the producer cultures revealed no contamination. Samples of the cultures from each medium were streaked onto BACa plate for deferred antagonism tests (P-typing of the cells from liquid cultures). P-types of 636 and 677 were obtained
for strain MPS and strain Mia respectively when growing in both non-supplemented dialysate THB and regular THB. A P-type of 676 was observed for strain MPS growing in supplemented dialysate THB, indicating an additional activity against standard indicator 4, which had not been evident previously.
Table 3.1.2.1: Production of salivaricin MPS-like activity by *S. salivarius* strains MPS and Mia using liquid growth media

<table>
<thead>
<tr>
<th>Medium</th>
<th>dialysate of THB&lt;sup&gt;a&lt;/sup&gt;</th>
<th>supplemented dialysate of THB&lt;sup&gt;b&lt;/sup&gt;</th>
<th>regular THB</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I₁</td>
<td>I₂</td>
<td>I₅</td>
</tr>
<tr>
<td>MPS 3 h</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>MPS 5 h</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mia 3 h</td>
<td>+&lt;sup&gt;c&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mia 5 h</td>
<td>++&lt;sup&gt;d&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

The regular Todd Hewitt Broth (THB) was prepared from Bacto Todd Hewitt Broth Base following the manufacturer’s instructions. The producer strains were pre-grown in regular THB liquid medium for 18 h (37°C, 5% CO₂) and a 1 ml aliquot was taken and inoculated into 16 mL of the corresponding THB medium.

- **a.** dialysate of THB was prepared using 8,000 Da cut off dialysis membrane tubing
- **b.** dialysate of THB was supplemented with 5% glucose, 0.05 M CaCO₃ and 5 µg.mL⁻¹ cysteine
- **c.** “+” indicates an inhibitory zone with radius about 0.8 cm from the centre of the well
- **d.** “++” indicates an inhibitory zone with radius about 1.0 cm from the centre of the well
3.1.3 Salivaricin MPS production using bi-phase medium

The inhibitory activity generated by the four tested strains is summarized in Table 3.1.3.1. Activity assays were carried out every two hours starting from 3 h of incubation of the producer culture until 7 h, plasmid-negative strain Mia and salA/salC negative (salA and salC encodes salivaricin A and salivaricin C respectively, both salivaricin variants are known to inhibit the growth of *S. pyogenes* indicators). Strain Mia controls revealed no inhibitory activity against any of the indicators, providing a strong indication that the anti-*S. pyogenes* activity observed from strains Mia and MPS did not come from the crude medium component of the liquid-phase of the medium, which may contain substances dispersed from the BACa surface during incubation.

No anti-*S. pyogenes* activity appeared to be produced by the plasmid-negative strain Mia. The inhibitory patterns of strain MPS and strain Mia against indicators I_2 and I_5 did not vary much during prolonged incubation; however, a decrease in the inhibitory zone size was observed from strain MPS against I_7, as the incubation duration increased. In comparison, the largest inhibitory zone from strain Mia on I_7 was observed at 5 h incubation time, and went on the same decreasing trend as found for strain MPS.

The anti-I_2 activity of both producers appeared relatively unchanged throughout the entire incubation period, the anti-I_5 activity increased somewhat beyond 3 h and 5 h respectively for strain MPS and strain Mia. The anti-I_7 activity of strain Mia at 3 h and 7 h of incubation time was similar in terms of inhibitory zone sizes, and stronger inhibitory activity was observed from the 5 h sample. The fluctuation of inhibitory activity during prolonged incubation is an example of negative feedback control, a molecular regulatory mechanism utilized by several families of lantibiotics, including the two-component sub-class of the type C lantibiotics such as cytolysin (Haas *et al.*, 2002). Depression of cytolysin biosynthesis occurs when the regulatory subunit
reaches an extracellular threshold concentration at a specific cell density (Haas et al., 2002). This finding provides another clue that at least some of the anti-I₇ inhibitory activity of strain MPS and strain Mia may correlate to the action of salivaricin MPS, a bacteriocin which was hypothesized to comprise a two-component system based on the genetic analysis of its producer S. salivarius strain MPS, and the biosynthesis of salivaricin MPS may be subject to a negative feedback control pathway.

The heat stability assay of all of the strains MPS and Mia samples having anti-S. pyogenes activity from 3 to 7 h incubation period showed no inhibitory activity against indicators I₂, I₅ and I₇ after heating at 80°C for 30 min. Loss of activity upon heating indicated the heat labile nature of the anti-S. pyogenes activity, most likely due to denaturation of the putative salMPS inhibitor, at high temperature. Salivaricin MPS, is a large, heat labile bacteriocin with a $M_r$ of 62 kDa based on back translation of the gene sequence, which is larger than most (if not all) known bacteriocins. With the exception of some proteins that have evolved thermo-stability, most large proteins having a molecular weight similar to that of salivaricin MPS lose their biological function at elevated temperatures due to denaturation.
Table 3.1.3.1 Production of salivaricin MPS-like activity by *S. salivarius* strains MPS and Mia using bi-phase medium

<table>
<thead>
<tr>
<th>Producer strain</th>
<th>3 h</th>
<th>5 h</th>
<th>7 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I$_2$</td>
<td>I$_5$</td>
<td>I$_7$</td>
</tr>
<tr>
<td>MPS</td>
<td>0.6$^a$</td>
<td>0.6</td>
<td>0.9</td>
</tr>
<tr>
<td>Mia</td>
<td>0.6</td>
<td>0.6</td>
<td>0.7</td>
</tr>
<tr>
<td>Mia plasmid$^{-b}$</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mia salA/salC$^{-c}$</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

(a) The size of inhibitory zones were measured as the radius (in cm) from the centre of the well
(b) Plasmid-negative *S. salivarius* strain Mia, negative control
(c) *salA/salC* genes negative (not able to produce salivaricin A or salivaricin C) *S. salivarius* Mia, negative control
3.2 Studies of salivaricin MPS-like inhibitory activity produced by other streptococcal strains

3.2.1 Screening of streptococci for salivaricin MPS-like anti-pyogenes activity

Although the spectra of *S. salivarius* strains MPS and Mia included inhibition of all four *S. pyogenes* indicators in the standard typing system used, the additional inhibitory activity towards indicators I₁ and I₄ somewhat broadened the inhibitory spectrum of salivaricin MPS. Even if the additional non-*S. pyogenes* specific inhibitory activity was from other inhibitory substance(s) released together with salivaricin MPS, this further complicates the development of an understanding of the inhibitory system.

One possibility raised was that both putative components of salivaricin MPS may not be necessary for the demonstration of anti-*S. pyogenes* activity, a prospect which means that instead of requiring both gene products (*salMPS* and *salMPSvar*), only one component may be necessary for inhibition of *S. pyogenes* strains. In order to address this possibility, various additional strains of streptococci capable of producing inhibitors were screened in an attempt to identify a candidate strain which was capable of producing a relatively narrow anti-*S. pyogenes* spectrum (i.e. P-type 226) and at the same time possessing the genetic potential for salivaricin MPS production (being PCR-positive for at least one of the two salMPS genes).

*S. salivarius* strains Min 5 and T18A were shown by PCR to yield PCR products for two primer sets, (MPS Blis P1) and (MPS Variant) which had been designed to amplify the two salMPS structural genes, *salMPS* (original gene) and *salMPSvar* (variant gene) from *S. salivarius* strain MPS (Table 3.2.1.1). Strain DC156a was the only strain to give a PCR product corresponding to the original, but not to the variant gene; while five other strains, including *Streptococcus uberis* strain NY42, yielded a PCR product corresponding only to the variant gene.
A P-type of 226 reflects *S. pyogenes*-specific inhibitory activity of the producer strain in the deferred antagonism test. Strains having P-types other than 226, such as Min5 (777) and Newall (657) or displaying variations in P-types in repeat tests (DC156a and Rebecca) were excluded from further study, since they were presumed to produce additional inhibitory activity.

The cross-immunity test established that the growth of strains NY42, Jim8771, Rebecca and Newall was not antagonized by the 636 P-type inhibitory activity of strain MPS. The insensitivity of these strains to *S. salivarius* MPS inhibitory activity is consistent with these strains harboring a common salMPS-specific immunity gene in their genomes. Specific immunity genes are usually coupled to the corresponding inhibitor-encoding gene(s) in the genome of bacteriocin-producing strains (Tagg, personal communication). The P-type 226 producer strains may have acquired the immunity gene of salMPS which protected them from the action of salMPS-like inhibitory agents and this provides a further indication of the presence of salMPS encoding sequences in their genomes.

Among all of the screened strains, only *S. salivarius* strains Jim 8771 and *S. uberis* strain NY42 displayed a consistent *S. pyogenes* specific pattern (P-type 226). *S. uberis* strain NY42 was one of very few non-*S. salivarius* 226 strains with *S. pyogenes* specific activity. It was also the only P-type 226 strain found to yield inhibitory activity upon urea extraction, a method which greatly decreased the haemoglobin content of its inhibitor-containing extract when compared to the freeze thaw approach that was required for other strains (Figure 3.2.1.2).
Table 3.2.1.1 The *salMPS* and *salMPSvar* content and P-types of potential producers of salivaricin MPS-like activity

<table>
<thead>
<tr>
<th>Strain</th>
<th>salMPS</th>
<th>salMPSvar</th>
<th>P-type *</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Streptococcus salivarius</em></td>
<td>MPS</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Mia</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Min5</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>T18A</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>#5</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>DC156A</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Jim8771</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Rebecca</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>Newall</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>Streptococcus uberis</em></td>
<td>NY42</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

* P-type results from repeat deferred antagonisms
Figure 3.2.1.2 Extraction of inhibitory activity from various *S. salivarius* strains and *S. uberis* strain NY42 using 7 M urea

Urea extracts from several producer stains were prepared by suspending producer culture lawns (grown on BACa for 21 h, at 37°C, 5% CO₂) in 3 mL of 7 M urea for 4 h at 4°C. An aliquot (30 µL) of each of the urea extracts from selected producer strains was spot assayed on CAB against *S. pyogenes* EB1.

Inhibitory activity against a lawn culture of *S. pyogenes* EB1 was detected from four of the producer strains tested:

*S. salivarius* strain Min5 (P-type 777), zone size ~0.5 cm in radius
*S. salivarius* strain T18A (P-type 777), zone size ~0.7 cm in radius
*S. uberis* strain NY42 (P-type 226, grown on CAB), zone size ~0.7 cm in radius
*S. uberis* strain NY42 (P-type 226, grown on BACa), zone size ~ 1.0 cm in radius
3.2.2 Factors potentially influencing the production and detection of salivaricin MPS-like activity

Ultraviolet sterilization of the agar surface in deferred antagonism tests

In preliminary tests, a decrease in inhibitory activity was observed for *S. salivarius* strain MPS in deferred antagonism tests when the assay plates were sterilized by UV irradiation treatment, instead of the conventional chloroform method. Inhibitory activity against indicator I₅ was not evident following UV treatment and the inhibition of indicator I₈ was much less than with use of chloroform (Figure 3.2.2.1 a). A similar absence of inhibitory activity was also evident in deferred antagonism tests of strains DC156a, Newall and Rebecca with use of UV-irradiation, and the latter two strains were P-type 000 as compared to 226 with use of chloroform sterilization. The underlying basis of this phenomenon remains unknown. One suggestion is that the UV-irradiation somehow causes damage to some amino acid residues of the inhibitor molecule resulting in disabling the interaction between the inhibitor and its target.

It appeared that the effect of UV varies for different *S. salivarius* strains. The inhibitory profile of strain MPS did not appear to be as severely affected by the UV irradiation step as strain Newall and strain Rebecca, since other than a reduction of activity against indicators I₅ and I₈, while the activities against the other sensitive indicators appeared unchanged, this may be due to the production of other inhibitor(s) which are not affected by UV. Alternatively, UV-irradiation may affect structural elements (such as secondary and tertiary structures) which promote biological function of the inhibitor in certain strains. Such damage would likely result in a partial reduction of the inhibitory activity, rather than complete loss of the spectrum. The subtle differences in the inhibitor putative active site from strain to strain may also account for variations in inhibitory profile after UV irradiation, as some of these may be more affected by UV than the others.
In addition, a combined UV irradiation-chloroforming experiment was conducted to help determine whether the inhibitory activity of some *S. salivarius* strains was dependent upon chloroform activation. Except for *S. salivarius* strain Mia and MPS, no inhibitory activity against any indicators was observed from any other *S. salivarius* strains (DC156a, Newall and Rebecca), after exposing the UV-irradiated (20 min) plates to chloroform (30 min). The inhibitory activity of *S. uberis* strain NY42 was also absent after the combined UV-chloroform treatment. Chloroform treatment of human serum has been reported to increase blood proteolytic activity by activating fibrinolysis (Rocha *et al.*, 1948), but no evidence could be found in the literature regarding an activating effect of chloroform on bacteriocins. The above observations are consistent with a bacteriocin-inactivating effect of UV-irradiation rather than a bacteriocin-enhancing effect of chloroform.

Addition of blood and saliva to growth media

In preliminary experiments designed to extract the putative salMPS inhibitor by the freeze-thaw method, it had been found that stronger inhibitory activity was produced when saliva was applied onto the surface of BACa agar prior to inoculation of the producer strain. This observation indicated that there might be a combined enhancement effect between blood and saliva for production and/or recovery of the salMPS-like activity. However, when this experiment was later repeated the addition of saliva no longer appeared to consistently enhance inhibitor recovery in freeze thaw extracts.

Although a salivaricin A-inducing component (Philip *et al.*, 2006) was shown to be present in human saliva after colonizing subjects with a salivaricin A-producer strain, the results indicated variations of producer cell numbers (per ml of saliva) rather unpredictably influenced the levels of salivaricin-inducing activity present in saliva. In the present study, it was difficult to address whether the introduction of saliva was beneficial for induction of inhibitor production under laboratory conditions using
saliva collected from a single subject. Further experiments with a larger sampling pool providing a variety of saliva activities could help assess the role of a potential inducing effect of saliva in a more systematic manner.

On the other hand, no anti-\textit{S. pyogenes} activity could be detected in the freeze thaw liquid if CAB (i.e. with no blood added) was used as the growth medium. From this observation, it appeared that the blood component of BACa may play an essential role rather than an enhancement effect in the inhibitor production. Controls established that blood and saliva separately did not cause any inhibitory effect. The presence of blood (as in subjects experiencing bleeding of the gums associated with periodontal disease) and saliva might provide important stimulatory factors in the natural oral environment of \textit{S. salivarius} MPS, encouraging enhanced production of salivaricin MPS activity.
Figure 3.2.2.1 The effect of UV, blood and saliva on the inhibitory activity of *S. salivarius* strain MPS

Strain MPS showed P-type of 636 (see Table 3.1.1.1 for more details on P-type calculation) using chloroform sterilization and a reduced P-type of 614 with use of UV irradiation. Deferred antagonism of strain MPS were assayed on BACa, producer strain (producer streak was removed before seeding indicators) was grown aerobically for 18 h at 37°C, with 5% CO₂.
Variations in the preparation of strain MPS freeze thaw extract using BACa or CAB media:

- **BACa-saliva**: producer lawn grown on BACa without introduction of saliva
- **BACa+saliva**: producer lawn grown on BACa with introduction of saliva
- **CAB-saliva**: producer lawn grown on CAB without introduction of saliva
- **CAB+saliva**: producer lawn grown on CAB with introduction of saliva

50 µL aliquot of the freeze thaw extract samples were well assayed on CAB for inhibitory activity against *S. pyogenes* indicators I<sub>7</sub>, I<sub>5</sub> and I<sub>2</sub> as labeled on each assay plate, inhibitory activity was only detected from free thaw extract samples when producer strain was grown on BACa with introduction of saliva.
3.3 Attempt to adsorb the prototype salMPS inhibitor to target cells

For many bacteriocins, the action of inhibition is initiated by binding to the target cell, promoting the idea of using *S. pyogenes* cells to bind the prototype salMPS inhibitor as part of a purification protocol. This concept was used by Dodd (Dodd, 1999) who mixed partially-purified salMPS with *S. pyogenes* I\textsubscript{7} cells and found that this appeared to remove the salMPS inhibitory activity from solution. On the basis of previous experience strain I\textsubscript{7} was considered the most sensitive indicator. However *S. pyogenes* EB1 has been used as an additional indicator since the beginning of this project due to its apparent high sensitivity. Strain EB1 was selected as an inhibitor-binding strain since (unlike strain I\textsubscript{7}) it does not itself produce any BLIS, minimizing the possibility of heterologous inhibitor interference. By mixing the active freeze thaw extracts with sensitive *S. pyogenes* cells, it was hoped that the prototype salMPS inhibitor could be bound to the cell surface and then subsequently be eluted using high ionic strength buffers.

BACa freeze thaw extracts of *S. uberis* strain NY42 showed an inhibitory activity of titre 4 (reciprocal of the highest dilution which still produces inhibitory activity) against *S. pyogenes* strains EB1 and I\textsubscript{7}. The observation that the anti-*S. pyogenes* inhibitory activity was completely removed upon mixing with EB1 cells (either viable or heat killed) and reduced to titre 1 (titre 4 before mixing) after mixing with viable I\textsubscript{7} cells indicated that the prototype salMPS inhibitor did indeed bind to the target *S. pyogenes* cell surface.

The ratio of cells to inhibitor required for complete binding of the inhibitor molecule to EB1 and I\textsubscript{7} cells is not known, and thus it is hard to assess the amount of inhibitor present in the supernatant (if any). Assay results indicated a saturated binding between the inhibitor and viable I\textsubscript{7} cells, as there was sufficient unbound inhibitor present in the supernatant to be detected by the inhibitory activity assay (titre 1 against EB1). An assumption could be made that given the same number of cells of EB1 and I\textsubscript{7}, a
smaller number of inhibitor molecules may be engaged in the complete inhibition of I₇ cells. Alternatively, I₇ was also known to produce dysgalacticin (bactericidal towards *S. pyogenes*, P-type 222), which may be released into the supernatant during the binding process and account for the weak anti-EB1 activity.

Binding with heat-killed EB1 and I₇ cells removed the inhibitory activity completely and indeed it appeared that the inhibitor bound more effectively to heat-killed cells than to live cells. This may be a result of altered cell surface integrity by heating, creating more recognition sites for the inhibitor molecules which otherwise would remain unbound to viable cells. On the other hand, dysgalacticin is also heat labile, and so by heating the I₇ cells, the anti-EB1 activity due to dysgalacticin would have been destroyed, which could then more specifically allow demonstration of complete binding of the salMPS activity to the I₇ cells. Although salMPS from strain MPS does not inhibit I₆ (*Lactococcus lactis*), an additional binding experiment using viable I₆ cells indicated unexpectedly that binding to I₆ cells may have occurred, as the supernatant did not have any detectable inhibitory activity against EB1 and I₇. The prototype inhibitor may be recognized as a misfolded protein (and subsequently degraded) by *Lactococcus lactis* surface protease HtrA, a housekeeping trypsin-like serine protease which is involved in growth regulation and cellular defense under stressed conditions (Foucaud-Scheunemann and Poquet, 2003).

Elution with NaCl (1 M or 2 M), or 7 M urea did not recover any inhibitory activity (against indicators EB1) from *S. pyogenes* (either viable or heat killed) cells that had been pre-mixed with the salMPS-containing freeze thaw extracts. NaCl (1M or 2M) or urea (7 M) controls (+inhibitor) showed no reduction of the inhibitory activity against the *S. pyogenes* indicators. These findings indicated that the inhibitor molecule may have bound to the target cells irreversibly and then be resistant to salt elution, or that the inhibitor may have lost its elutable activity subsequent to binding either due to degradation or to uptake into the cell. However, by suspending the inhibitor-cell binding complex in high salt solutions, and subsequently assaying the supernatant for
inhibitory activity, the inhibitor (if successfully eluted) would have been required to interact with its target cell in a relatively high ionic strength environment. Although controls (1:1 dilution of active freeze thaw extract with high salt solution) indicated this did not substantially reduce inhibitory activity it is possible that the inhibitory activity of the molecule as recovered from its state of attachment to the substrate bacteria has been reduced to an undetectable level.

3.4 Purification of prototype salMPS inhibitor from strain NY42 using HPLC

3.4.1 Inhibitory activity of ammonium sulfate fractionated material

The precipitate of the NY42 BACa freeze thaw extract obtained after 50% saturation with ammonium sulfate had a titre of 32 against *S. pyogenes* strains EB1 and a titre of 16 against I7 (Figure 3.4.1.1). This represented a 4-fold increase in titre compared to that of the initial freeze thaw extract (titre 8 and 4 respectively against the two indicators). The inhibitory activity of the ammonium sulfate-precipitated material was inactivated by heating at 80°C, for 30 min. The activity spectrum corresponded to the 226 P-type of *S. uberis* NY42 (i.e. it was inhibitory only to the *S. pyogenes* indicators [I2, I5, I7 and I8] of the nine standard P-typing indicators). This was a promising result indicating that the concentrated activity may correspond to the same inhibitory agent present in the starting NY42 freeze thaw extract, which was hypothesized to contain the putative salMPS inhibitor.

However, considerable total activity was lost during this process. The initial extract had a total AU (activity unit) of 1296, with only 160 AU recovered after precipitation, indicating an activity recovery of just 12.3%. Previous purification studies (Dodd, 1999) showed ammonium sulfate at 50% saturation precipitated salMPS inhibitory activity from *S. salivarius* strain MPS cultures grown in chemically defined medium (CMD) with a recovery of over 60%. However, it was not known whether use of BACa as the growth medium and *S. uberis* NY42 as the producer would result in a
different ammonium sulfate concentration requirement to achieve comparable recovery. Alternatively, it is possible that the inhibitory activity from strain MPS may represent a mixture of the two MPS forms (from two salMPS genes) while with strain NY42, only one gene product is produced.

No inhibitory activity was detected in the 50% ammonium sulfate supernatant fluid. It was noticed that the colour of the supernatant was purple red, indicating a high content of haemoglobin, which has similar molecular weight and pI to salivaricin MPS.

Precipitation at 80% saturated ammonium sulfate concentration did achieve a higher recovery of total activity (78%). Salivaricin MPS is known from previous studies to remain active in ammonium sulfate (Dodd, 1999), therefore the loss of inhibitory activity in 50% saturated ammonium sulfate supernatant was unlikely to be due to denaturation of the inhibitor at high salt concentrations. This observation may be due to the putative salMPS produced by S. uberis NY42 being somehow different to its counterpart produced by S. salivarius MPS, in terms of the inhibitory activity in high salt environment.
The 50% saturation ammonium sulfate precipitate of NY42 freeze thaw extract was re-dissolved in a small volume of PBS, pH 7.0. Serial dilutions were prepared with sterile water and a 30 µL aliquot from each of the diluted samples was spot assayed on CAB medium. Inhibitory activity against EB1 and I7 showed titre of 32 (a) and 16 (b) respectively.

Although a better recovery of inhibitory activity was achieved with 80% ammonium sulfate saturation. The 50% saturation ammonium sulfate precipitate material was chosen for HPLC fractionation as the total protein composition was presumably less complex and therefore the HPLC fractionation maybe more efficient.
3.4.2 Gel permeation HPLC of precipitated strain NY42 freeze thaw extract

A discrete region of three active fractions (Figure 3.4.2.1) was obtained from gel permeation HPLC. The inhibitory activity of all three active fractions was heat labile and matched the P-type 226 spectrum of strain NY42 with strong inhibitory activity against EB1 and I7, and no activity was detected against I1 and I6 (not shown in Figure 3.4.2.2), indicating the presence of a heat-sensitive *S. pyogenes*-specific inhibitor. As such properties also are associated with salivaricin MPS, it was logical to presume that a putative salMPS-like inhibitor was being produced by NY42, and was responsible for the inhibitory activity detected.

Fraction 18 contained the strongest inhibitory activity. Weak inhibitory activity was also detected in fraction 20 against both EB1 and I7. A light red-brown pigmentation was evident in all fractions displaying inhibitory activity (17, 18 and 19); the coloration of which was most intense for fraction 18. Such coloration came from human haemoglobin (blood agar) as confirmed by the MS results (Table 3.4.4.1) of the in-gel digested protein band (Figure 3.4.3.1). This result further supported the idea that salivaricin MPS has a similar *M*$_r$ (62 kDa) to that of the haemoglobin tetramer (64 kDa).

Control activity assays established that human blood was not inhibitory to the salMPS sensitive-*S. pyogenes* indicators. Although the red-brown colour associated with the putative salMPS activity was observed frequently, it was not a requirement for such activity to be detected since a colourless putative salMPS active sample could also be achieved by 7 M urea extraction of intact NY42 cells. Unfortunately the inhibitory activity of the urea extract (titre 4 against EB1) was not as strong as the freeze thaw extract (titre 8 against EB1), and the inhibitory activity of the urea extract was more readily lost after 72 h of storage at 4°C.
Figure 3.4.2.1 Gel permeation HPLC chromatography of ammonium sulfate fractionated NY42 BACa agar freeze thaw extract material

HPLC column: Superdex 200 10/300 GL
Injector system: Applied Biosystem, 140A Solvent Delivery System
Running buffer: 20 mM HEPES, 150 mM NaCl, pH 7.2

The HPLC system was operated at a flow rate of 0.5 mL.min\(^{-1}\), each fraction was collected for 1 min (fraction size: 0.5 mL), the first fraction was collected at 15 min after injection of sample, and stopped at 45 min. The bar indicates fractions where inhibitory activity was detected.
One potential issue relating to the HPLC fractionation is that the active agent loaded on the column could potentially require the interaction of more than one protein. If so, then separation of these proteins (i.e. elution into different fractions) may cause loss of activity. Aliquots of the fractions corresponding to the other absorption peaks which did not show any inhibitory activity when assayed individually were mixed with an aliquot from fraction 18. No enhancement of inhibitory activity was detected in these mixtures which indicated that the salMPS-like activity from fraction 18 did not require any additional co-factor and that its activity was most likely due to a single inhibitor molecule.
Figure 3.4.2.2 Activity of HPLC-fractionated ammonium sulfate precipitated freeze thaw extract of *S. uberis* NY42

(a) (b) (c) (d)

Ctrl – re-dissolved NY42 freeze thaw extract (see Materials and Methods section 2.8)

A 30 µL aliquot of each HPLC fraction was spot-assayed on CAB medium against the indicators EB1 or I7 as labeled on each plate. The assay plates were incubated aerobically (5% CO2) at 37°C for 18 h, inhibitor activity was detected in fractions 17, 18 and 19 against both EB1 (a) and I7 (b). Slight inhibitory activity was also detected from peak-mix (mixture of peak fractions) against EB1 (c) and I7 (d). Assay of total mix (20 µL of each fraction) detected no inhibitory activity.
3.4.3 1D-SDS-PAGE of inhibitory fractions from gel permeation HPLC

Aliquots (2.5 µL and 12.5 µL) of selected fractions from the gel permeation HPLC were analyzed by 1D-SDS-PAGE (Figure 3.4.3.1) to detect protein components present for subsequent analysis and to be analyzed by MS. Lanes 2 – 7 were loaded with 2.5 µL of each HPLC fraction prepared in sample buffer. Lanes 10 – 15 were loaded with 12.5 µL of the fractions equivalent to those in lanes 2 – 7, and thus the in-gel band patterns of lanes 10 – 15 were more intense than that of lanes 2 – 7.

Lanes 2 and 3 showed multiple intense bands within the $M_r$ range 50 – 160 kDa corresponding to HPLC fractions 14 and 16. The two most intense bands in lane 2 were in the vicinity of 50 kDa and between the 20 and 30 kDa marker positions. Lanes 6 and 7 contained fewer bands, of mainly lower $M_r$ and intensities compared to those in lanes 2 to 5. The overall in-gel banding pattern across six sample lanes showed a reduction in protein content and potentially in the later HPLC fractions compared to the early fractions as expected. It was not possible to correlate a particular protein band with the inhibitory activity detected in fraction 18 (and 19). It was decided to perform in-gel digestion and MS analysis of hydrolysed and extracted peptides of sections of the gel lane to obtain more information about the sequence and search for possible homology with any known protein from online databases.

An intense band of $M_r$ ~16 kDa was observed for the two active fraction preparations (lanes 4 and 5). MS analysis of an in-gel protease digest of this band confirmed that this band contained human haemoglobin which was denatured by SDS to its monomeric form ($M_r = 1.7$ kDa). One unique band was also observed in lanes 4 and 5, with an estimated $M_r$ of 35 kDa, about half the size of the 62 kDa salMPS, which may correspond to a salMPSvar gene product of strain NY42.
Figure 3.4.3.1 SDS-PAGE of gel permeation HPLC active fractions of strain NY42 freeze thaw extract

Lane 1 (and 9): Novex Sharp prestained standard marker, (Invitrogen) \( M_r \) in kDa are labeled on the left of each band

Lane 2 (and 10): Fraction 14; 2.5 \( \mu \)L (12.5 \( \mu \)L)

Lane 3 (and 11): Fraction 16; 2.5 \( \mu \)L (12.5 \( \mu \)L)

Lane 4 (and 12): Fraction 18; 2.5 \( \mu \)L (12.5 \( \mu \)L)

Lane 5 (and 13): Fraction 19; 2.5 \( \mu \)L (12.5 \( \mu \)L)

Lane 6 (and 14): Fraction 21; 2.5 \( \mu \)L (12.5 \( \mu \)L)

Lane 7 (and 15): Fraction 23; 2.5 \( \mu \)L (12.5 \( \mu \)L)

Lane 8: blank

20 \( \mu \)L loading samples of the selected HPLC fractions of NY42 extract were prepared with 5 \( \mu \)L of NuPAGE sample buffer (Invitrogen), and loaded on a premade NuPAGE
4-12% gel (Invitrogen). The gel was run at 115 V, for 1 h, using 1 x NuPAGE MES SDS running buffer (with 500 µL of antioxidant added to the upper chamber). The gel was stained with 20 mL of SimplyBlue SafeStain (Invitrogen), and subsequently destained with 2 % (v/v) NaCl.

The region between the two long solid lines of lanes 12 and 13 was divided into 4 sub-sections (frac 1, 2, 3 and 4 of lane 12 and 13) and excised for in-gel digestion and mass spectrometry analysis of extracted hydrolysed peptides, followed by analysis of the MS data by database search.

3.4.4 Mass spectrometry results of inhibitor-positive gel permeation HPLC fraction on SDS-PAGE

A brief summary of the database search results of the MS/MS sequences is shown in Table 3.4.4.1. The search on the SwissProt database generated a large number of matches with human blood proteins, which indicated a limitation of using the BACa as the production medium for the freeze thaw inhibitor extraction method. The amount of human blood protein matches surpassed the matches for bacterial proteins by several orders of magnitude.

Assuming that the putative salMPS has the similar molecular mass (62 kDa) as that of salivaricin MPS produced by strain MPS, digested sequences from in-gel bands with apparent sizes around 60 kDa did match a periplasmic amino acid binding protein of streptococcal origin, which may correspond to target recognition and membrane insertion motifs of known bacteriocins, including several salivaricin variants. However, the lack of a salivaricin peptide library in the streptococcal database made this finding much less significant and inconclusive. Little information can be obtained from data searching with the current online database at this point, though there were trends suggesting possible matches, most results were rather trivial.
PCR testing indicated that *S. uberis* NY42 contained the salivaricin variant gene (*salMPSvar*, Table 3.2.1.1). This finding was supported by the sequencing matching results using a user-defined sequence library with multiple hits between the query sequences and salMPSvar sequence. This result further reinforced the theory that the two component system characteristic of *S. salivarius* strain MPS may not be necessary for the *S. pyogenes*-specific inhibitory activity, as the data indicates no sequence matches with salivaricin MPS were found.

Multiple matches were found between the query sequences and the translated salMPSvar sequence provided from the user-defined sequence, the rank peptide which matched the salMPS exactly contains ten residues (RVSIKVEPKM, score 10). In addition, a fifteen residue long peptide (RALGNGAQWGATARG, score 12) was also matched with the salMPSK12 variant (salivaricin MPS from *S. salivarius* K12). Both matches were considered significant hits by the searching algorithm, which defines matches with individual ion scores > 4 for identity or extensive homology (p<0.05).

Although both sequence matches with salMPSvar and salMPSK12 scored a high enough value to be considered as the significant hit, like salivaricin MPS, salivaricin MPS variant is a large protein with more than 500 residues, so the possibility of random matches with short peptide sequences can not be rule out.
### Table 3.4.4.1 Database search results of MS/MS sequences from the SDS-PAGE gel

<table>
<thead>
<tr>
<th>No. of Query</th>
<th>SwissProt</th>
<th>NCBI Strepto</th>
<th>User*</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1 (3574)</td>
<td>Mostly human blood proteins: serum albumin, hemoglobin subunits, keratin variants and Ig heave and light chains; G-proteins, co-factors and complement factors</td>
<td>ABC transporters, periplasmic components, putative glucosyltransferase, permease and other hypothetical proteins</td>
<td>SalMPSvar, cys dioxygenase, putative cell surface protein S. pyogenes</td>
</tr>
<tr>
<td>F2 (4318)</td>
<td></td>
<td>periplasmic amino acid binding protein, glycosyl transferase, pneumococcal histidine triad protein, translation initiation factor</td>
<td>SalMPSvar, ATP synthase subunits Bacillus sp.</td>
</tr>
<tr>
<td>F3 (4096)</td>
<td></td>
<td>uncharacterised ABC transport system, ATP binding proteins, transmembrane proteins, Bcl-2 family protein</td>
<td>SalMPSvar, putative cell surface protein S. pyogenes: β-galactosidase E. coli</td>
</tr>
<tr>
<td>F4 (3874)</td>
<td></td>
<td>putative aa ABC transporter, DNA topoisormerase, hypothetical protein gbs1149, putative periplasmic component.</td>
<td>SalMPSK12var**</td>
</tr>
</tbody>
</table>

* Only matches with the bacterial protein were listed

** SalMPS variant produced by S. salivarius strain K12

NCBI Strepto refers to streptococcal database
3.4.5 Attempts to remove the haemoglobin content of the freeze thaw extract samples using urea and GuHCl

The colour of haemoglobin acted as an additional indicator in HPLC, providing a visual indication of the approximate location of the inhibitory activity, although the presence of haemoglobin is undesirable for purification purposes. Preliminary attempts to remove the haemoglobin from the preparation with urea and guanidine hydrochloride (GuHCl) did not succeed: GuHCl dissociated haemoglobin quite effectively at concentrations above 4 M, as indicated by a fading of the red colour due to dissociation of the haem from the globin protein (Figure 3.4.5.1), but the inhibitory activity was also destroyed, inhibitory activity could not be recovered after dialysis of the GuHCl containing samples.

Urea (8 M) did not dissociate the haemoglobin and had no effect on inhibitory activity. Although both GuHCl and urea failed to accomplish the goal of dissociating haemoglobin while retaining the inhibitory activity the findings were supported by the literature which indicates GuHCl is a more potent denaturing agent than urea, bringing about dissociation of the haemoglobin tetramer into individual $\alpha$- and $\beta$-globin chains (Kawahara et al., 1965).
Figure 3.4.5.1 Dissociation of haemoglobin by GuHCl

From left to right, 200µL of strain NY42 BACa freeze thaw extract in distilled water (first tube), and 1 M to 6 M GuHCl. No inhibitory activity could be detected from mixtures containing GuHCl at concentrations of $\geq 4$ M.
3.5 Preliminary ion-exchange chromatography fractionation of NY42 extract containing inhibitory activity

Figure 3.5.1 Inhibitory activity of desalted NY42 freeze thaw extract after ammonium sulfate precipitation

Re-dissolved 80% saturation ammonium sulfate precipitate of NY42 freeze thaw extract was desalted with MilliQ water (Materials and Methods, section 2.9) in order to minimize its ionic strength which would otherwise interfere with the binding process during FPLC fractionation.

The exchange process was repeated three times following spot assay (30 µL aliquot) on CAB medium against *S. pyogenes* EB1, inhibitory activity of titre 16 were obtained as indicated by the clear zone (dark coloured circular zone) observed for 1:16 dilution sample, this preparation was then used as the loading sample for FPLC.
Ammonium sulfate precipitation was effective in initial fractionation of the complex protein mixture from crude samples such as the NY42 freeze thaw extract. Sample concentration could also easily be achieved by re-dissolving the precipitate in a small volume of buffer. Ammonium sulfate precipitation at 50-80% saturation achieved a higher (80%) AU (activity units) recovery compared to the previous attempts with 0-50% ammonium sulfate saturation. The titre of the inhibitory activity was used as an indication of the amount of putative inhibitor that was present in the sample. The re-dissolved ammonium sulfate precipitate of NY42 freeze thaw extract showed inhibitory activity against EB1 of titre 16, which was considered suitable so that the inhibitory activity could be detected during ion-exchange chromatography.

3.5.1 Ion-exchange column binding test of NY42 freeze thaw extract

Aliquots of desalted ammonium sulfate fractionated inhibitory activity were manually injected onto two different ion-exchange columns to investigate which was more suitable to bind the putative salMPS inhibitory activity. Ion-exchange chromatography using a CM ion-exchange column did not result in any inhibitory activity against EB1 being detected in any of the high salt wash fractions, but inhibitory activity was detected in the effluent (direct flow-through) fraction (Figure 3.5.1.1 [a] and [b]). Ion-exchange using DEAE gave no inhibitory activity in the direct flow-through fraction. Inhibitory activity was detected only in fraction 1 (minor activity from fraction 2) of the high salt wash (Figure 3.5.1.1 [c] and [d]).
CM (DEAE) column was first washed with high salt buffer B (Material and Methods, Table 2.9.1) and followed by washing again with buffer A prior to sample loading. Effluent during the two buffer washing steps was collected (column wash B and column wash A) and spot assayed (30 µL aliquot) for inhibitory activity. 10 mL of desalted NY42 freeze thaw extract was then injected manually on to the CM (DEAE) column with a flow rate of approximately 1 mL.min⁻¹.
Effluent collected during sample injection and low salt buffer wash was designated as the flow-through and wash unbound fraction. One mL fractions (F1 – F6) were collected from the high salt buffer wash of the CM (DEAE) column. Aliquots (30 µL) of each of the collected fractions (and the flow-through and wash unbound fraction) were spot assayed on CAB medium against EB1, and all assay plates were incubated aerobically (5% CO₂) at 37°C, for 18 h. An aliquot of the loading sample was also assayed for inhibitory activity as the control.

Inhibitory activity was detected from the flow-through fraction of CM column (a) but not that of DEAE column (c), no inhibitory activity was detected from the two buffer washing effluent fractions nor from the wash unbound fraction. No active fraction was obtained from high salt buffer wash of the CM column (b), however, fraction 1 from the high salt buffer wash of the DEAE column showed inhibitory activity against EB1 (d).

The column-binding data indicated that the inhibitory activity did not bind to the CM column but did bind to the DEAE column and was subsequently eluted off using high salt buffer. Salivaricin MPS from strain MPS has a predicted pI value of 6.5 (Wang, 2007). Assuming that the putative salMPS inhibitor from strain NY42 has the same or similar pI value to that of salMPS, the column binding results demonstrated that under basic pH (pH 8.0) conditions the putative inhibitor is negatively charged, and binds to the anion-exchanger (DEAE) resin more readily, while at acidic pH (pH 6.0), little interaction occurred between the putative inhibitor and the CM cation exchanger resin.

Although the inhibitory activity appeared not to bind to the CM resin, passage through this matrix may help effect separation of haemoglobin from the inhibitor molecule. The dark red colouration deposited onto the CM column by passage of the sample was resistant to a high salt buffer wash, indicating irreversible binding between much of the haemoglobin and the ion-exchange resin. Hence, by passing the load material
though the CM column, a more purified active fraction was obtained with much of the haemoglobin removed. However, it is important to note that the flow-through fraction was not completely colourless, as the resin in the CM column may have been saturated with haemoglobin.

Discolouration and reduction in inhibitory activity was observed when the NY42 freeze thaw extract was mixed with free CM fast flow Sepharose. One possible explanation for why such binding was not observed from the CM column binding test could be that the CM ion-exchange column may become saturated with excess inhibitor molecules and the inhibitory activity observed in the effluent fraction may be from unbound inhibitor molecules. If this is indeed the case, results from both the CM column binding test and free CM Sepharose binding indicated an irreversible binding event between the inhibitor molecule and the ion-exchange resin, as almost no inhibitory activity could be recovered by washing the CM column with high salt buffer.
3.5.2 Fast protein liquid chromatography

Figure 3.5.2.1 (a) FPLC of the CM column effluent material from the column binding test
(b) FPLC of desalted 80% ammonium sulfate precipitated NY42 freeze thaw extract
For each chromatography run of the FPLC, a 2 mL sample of inhibitor activity was injected on to the ÄKTAprime plus system (at 0 min) to which was connected the DEAE column. The system was operated with a flow rate of 1 mL.min$^{-1}$, and 20 mM Tris, pH 8.0 was used as the low salt buffer (buffer A) and the same buffer containing 2 M of NaCl was used as the high salt washing buffer (buffer B). Y-axis is the UV absorbance (blue curve) in mAu at 280 nm, and X-axis is time in min (with fraction number labeled above it). The green and red curves on each chromatography represent the salt concentration and conductivity respectively. High salt buffer elution started at break point 3 with 0% buffer B and ended at break point 4 (30% buffer B). One mL fractions were collected and aliquots were spot assayed on CAB against EB1 for inhibitory activity. The column was washed with 100% buffer B (break point 4-7) before the end of each FPLC run (break point 9).

FPLC using the same DEAE column but with the 80% ammonium sulfate precipitated NY42 freeze thaw extract (after dialysis with MilliQ water) did not detect any active fractions from high salt buffer elution, but most of the inhibitory activity was observed from the effluent fractions (first three fractions) collected during sample injection. Insufficient buffer exchange of the precipitated NY42 freeze thaw extract which leads to relatively high content of ammonium sulfate in the loading sample was probably responsible for this result, as an elevated conductivity associated with high UV absorbance (marked by the bar in [b]) was observed on chromatography during sample injection, and hence little binding occurred between the inhibitor and the column.

Inhibitory activity against *S. pyogenes* EB1 was detected from fraction 13 (indicated by the bar in [a]) after FPLC of the CM column effluent material (from the column binding test). As expected, a red-brown colour was observed, but of much reduced intensity compared to the colouration of the previous HPLC-active fractions. Both the 226 P-type inhibitory spectrum and heat lability of the inhibitory activity from fraction 13 matched that of salivaricin MPS, indicating the presence of a putative salMPS-like inhibitor and validating the capability of FPLC to separate such activity from unrelated material in crude preparation. However, the presence of haemoglobin in the active fraction remains a challenge and is most likely due to the similarity of pI between haemoglobin (pI 6.9) and the putative salMPS inhibitor (predicted pI 6.5).
Chapter 4

Conclusions

Previous characterization of salivaricin MPS had discovered several unique features of this macromolecule compared to the bacteriocins produced by other members of the *S. salivarius* family. The most notable characteristic was the relative fast induction of production of inhibitory activity with *S. pyogenes* specificity of salivaricin MPS, which made its producer *S. salivarius* strain MPS an ideal candidate as an oral probiotic. A two-component inhibitor system associated with salivaricin MPS inhibitory activity was also established based on initial sequence analysis, two genes have been identified in the strains MPS genome which are thought to encode for two similar salMPS peptides. The initial aim of this project was to purify salivaricin MPS using strain MPS as producer and investigate its mode of action regarding the two-component system. However, the recovery of salivaricin MPS from *S. salivarius* strain MPS had presented several challenges: the greatest difficulty came from the inability to reproducibly obtain the inhibitory activity from cultures in liquid media. It was difficult to address the underlying cause for this inconsistency, but our study did show several contradictory findings regarding the methodology of previous inhibitor-production experiments, which should be re-assessed in greater depth.

Difficulties encountered with strain MPS switched the focus of this project to look for alternative potential salMPS producers. Indicated by the screening study, *S. uberis* strain NY42, proved to be a more reliable producer with a more specific anti-*S. pyogenes* inhibitory activity and less complicated genetic composition (i.e. single salMPS gene) compared to strain MPS. Much of the study of strain NY42 relied on the extraction of inhibitory activity from producer growth cultures, freeze-thaw extraction followed by conventional ammonium sulfate precipitation was capable of enriching the inhibitory activity to a sufficient level; however, the separation of blood proteins, especially haemoglobin from the crude material while maintaining inhibitory
activity remains the biggest challenge for purification of the putative salMPS inhibitor. Extraction of inhibitory activity from strain NY42 culture lawn using urea could greatly reduce haemoglobin content, but the apparent loss of inhibitory activity during prolonged storage made the use of such extraction methods less applicable for chromatography studies.

Although reproducible production of the inhibitory activity from *S. salivarius* MPS was not successful, further characterization of the salMPS-like inhibitory activity was addressed including the apparent UV-sensitivity and blood requirement for production and/or action. This has provided more information about the nature of the inhibitor, as well as its producer strains. It is important to appreciate that the production of bacteriocin(s) under optimal laboratory conditions, represent only one possible way of simulating *in vitro* the *in vivo* production environment for the producer strain. As there is almost nowhere in nature that one bacterium grows as a single organism, interaction with other bacteria may play a crucial role in the induced production of certain bacteriocin(s). Such interactions are unlikely to occur when production tests are carried out in the laboratory, with pure cultures and contamination is avoided.

Nevertheless the activity of *S. uberis* strain NY42 as a putative salMPS producer has been supported by this study, yet more knowledge and experiments are essential to further evaluate its inhibitor production pathway. It is anticipated that the purification of the putative salivaricin MPS from strain NY42 and confirmation of its identity will lead to more insight into the role in nature of this novel inhibitory macromolecule and its potential applications in human medicine.
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