Investigation into the factors that allow colonization and infection with the organism *Streptococcus pneumoniae*

Ahmed Abdulfattah

The thesis is being submitted for the degree of PhD

The thesis is being submitted to the University of Glasgow;

The college of Medical, Veterinary and Life sciences

May/2023

Table of Contents

List of Figures ..... **vii**

List of Tables **x**

Acknowledgement ..... **xi**

Abstract **xii**

List of Abbreviations ..... **xiv**

CHAPTER 1: Introduction and Literature Overview **1**

1.1 Bacteriology and Epidemiology of *S. pneumoniae* **1**

1.2 Literature Review **6**

1.2.1 Colonisation and Invasion 6

1.2.2 Pathogenesis and Virulence of S. pneumoniae 11

1.2.3 Diagnosis and Treatment 14

1.2.4 Antimicrobial Peptides (AMPs) 20

1.2.4.1 Bacteriocins 20

1.2.4.1.1 Classification of bacteriocins. 21

1.2.4.1.2 General functions of bacteriocins. 25

1.2.4.1.3 Mechanism of action of bacteriocins against competing organisms 29

1.2.4.1.3.1 Bactericidal action through pore formation 29

1.2.4.1.3.2 Inhibition of cell wall biosynthesis 29

1.2.4.1.3.3 Inhibition through binding of DNA 30

1.2.4.1.3.4 Inhibition through disruption of ATP synthesis 30

1.2.4.2 Fratricide 31

1.2.5 Bacteriocin production in S. pneumoniae 31

1.2.6 The blp locus in S. pneumoniae 32

1.2.7 Competence regulatory system in S. pneumoniae 36

1.2.8 Competence induction 37

1.2.9 Interplay between competence regulatory system and bacteriocin production 39

1.3 Thesis Aims and Objectives 42

CHAPTER 2: Materials and Methods 45

2.1 Strains and Plasmids 45

2.2 Chemicals 46

2.2.1 Growth Medium 46

2.2.2 Enzymes 46

2.2.3 Bacterial Strains Growth Conditions 46

2.3 Bacterial Stocks Storage 47

2.4 Streptococcal Culture 48

2.5 Gram Staining 48

2.6 Catalase Test 49

2.7 Optochin (OP) Sensitivity Test 49

2.8 Bile Solubility Test 49

2.9 Antibiotic Susceptibility Test 50

2.10 Bacteriocin Induction 50

2.11 Overlay Assay 50

2.12 Overlay Assay (Modified test) 51

2.13 Synthesis of BlpCR6 bacteriocin inducer 52

2.14 Molecular method 52

2.14.1 Species-specific PCR assays 52

2.14.2 Genetic Pneumococcal differentiation.....53

2.14.3 Genomic DNA extraction.....53

2.14.4 Extracting genomic DNA from clinical isolates and specimens 54

2.14.5 Purification of DNA 55

2.14.5.1 Bacterial Cell Lysis.....55

2.14.5.2 Precipitation of DNA. 55

2.14.5.3 Storage of DNA. 56

2.14.6 Standardized Polymerase Chain Reaction (PCR).....56

2.14.7 Agarose Gel Electrophoresis 58

2.15 DNA Cloning into Plasmid Vectors.....58

2.16 Cloning and Expression of BlpMNO 59

2.17 Restriction Digest.....59

2.18 Gel Extraction and PCR Purification.....59

2.19 Ligation Reactions.....60

2.20 NEBase Changer Reaction.....60

2.21 Construction of the Sweet Janus (SJ) Cassette for the BlpMNO Gene Knock Out 60

2.22 Transformation of *S. parasanguinis*.....61

2.23 Liquid Inhibition Assay (Tube Method).....61

2.24 Bacterial strain identification VITEK 2 62

CHAPTER 3:The Role of Blp Locus in *S. PNEUMONIAE* TIGR4 Bacteriocin Production 63

3.1 Introduction 63

3.2 Bacterial Growth Phase 64

3.3 Results **65**

3.3.1 Bacteriocin activity in S. pneumoniae 65

3.3.2 The effect of pH (slight acidity-pH 6 versus slight alkalinity pH 8) 70

3.3.3 Inhibition assay 71

3.3.4 DNA Sequencing 73

3.4 Discussion **75**

3.5 Conclusion **80**

CHAPTER 4: Cloning and Expression of a Putative Immunity Protein from TIGR4 .. **81**

4.1 Introduction to the Function and Mode of Action of Immunity Proteins of Class II bacteriocins **81**

4.2 Overview of TIGR4 ..... **81**

4.3 Why Plasmid and Cloning Is Required **83**

4.4 Results **85**

4.4.1 Modifications of the pBAVIK1-T5 85

4.4.2 BlpMNO amplification and uptake in pBAV-Tag 90

4.4.3 Rectifying the Immunity Protein Mutation Through Site Directed Mutagenesis 93

4.4.4 Transformation of the TIGR4 Wildtype and Other Streptococcus Strains 94

4.4.5 Site Specific Structural Predictions for N Terminal and the C terminals of Recombinant Protein 96

4.5 Discussion **98**

4.5.1 Function of bacteriocins in S. pneumoniae and Their Similarity to Antibiotics as Observed in This Investigation 98

4.5.2 Plasmid Properties That Made It Ideal for Use in The Investigation 98

4.6 Conclusion **100**

CHAPTER 5: Genetic Basis of Bacteriocin-Resistant *S. parasanguinis*..... 102

5.1 Introduction 102

5.2 Results..... 103

5.2.1 Co-culture of TIGR4 strain with S. parasanguinis in BHI medium 103

5.2.2 Confirmatory Resistance Test After Liquid Tube Assay..... 104

5.2.3 DNA Extraction..... 105

5.2.4 Quality of DNA sequencing 106

5.2.5 Checking the Origin of The Sequences 111

5.2.6 Assembly of the WT Sequences to Produce A 'Reference' 112

5.2.7 Annotation 118

5.2.8 Comparing the Three Mutants to the Reference Genome 119

5.2.9 Discussion..... 120

5.2.10 Conclusions 123

CHAPTER 6: The Role of Metal Ions in Pneumococcal Bacteriocin Inhibition..... 125

6.1 Introduction 125

6.2 Results..... 128

6.2.1 Co-culture of TIGR4 Strain and Different Closely Related Pneumococci in Full Media129

6.2.2 Co-culture of TIGR4 strain and Streptococci Species in Metal Ions Depleted Media... 130

6.2.3 Co-culture of TIGR4 and Closely Related Streptococcus Species at Different Concentrations of Ca^{2+} ions..... 131

6.2.4 Co-culture of TIGR4 strain and closely related Streptococcus species at different Mg^{2+} concentration 136

6.2.5 Co-culture of TIGR4 and closely related Streptococcus species at different Mn^{2+} concentrations..... 140

6.2.6 Co-culture of TIGR4 and closely related Streptococcus species at different Fe^{3+} concentrations 144

6.3 Discussion and Conclusion 148

CHAPTER 7: General Discussion and Conclusion 159

7.1 Summary of Findings 159

7.2 Implications of pneumococcal bacteriocins for clinical application 164

7.3 Conclusion, Future Work and Perspective 164

References 166

List of Figures

Figure 1-1: The cell envelope Gram-positive bacteria. the cell envelope consists three layers. 2
Figure 1-2: A schematic illustration of the quorum-sensing system and its controlled cooperative activities in Streptococcus mutans. 36
Figure 1-3: The ComABCDE quorum sensing (QS) pathway of S. pneumoniae (Shanker and Federle, 2019). 38
Figure 1-4: The competence and Blp regulation in S. pneumoniae (Wang et al., 2018). 40
Figure 3-1: Blp locus of TIGR4 showing all the conserved genes that encode a typical two-component regulatory system (BlpR and BlpH). 64
Figure 3-2: The figure shows the Bacterial growth curve\Kinetic Curve. 65
Figure 3-3: Construction of Sweet Janus (SJ) cassette in the blp locus of TIGR4 strain. 66
Figure 3-4: The stabbing of strains TIGR4 and TIGR4 KO into TSA plate and incubation. 68
Figure 3-5: Bacteriocin-like activity in S. pneumoniae. 69
Figure 3-6: Overlay assay of TIGR4 and P4 as the producer strain and S. S. parasanguinis and P1104 as the reporter strain at pH 6 and pH 8 71
Figure 3-7: Representative growth curves of TIGR4 in cultures treated with CSP or without (control). 72
Figure 3-8: Representative growth curves of S. parasanguinis (Negative control), TIGR4 vs KO (positive control) and TIGR4 vs S. parasanguinis at different time points. 73
Figure 4-1: Shows the pBAV1K-T5 plasmid before digestion with EcoR1 and Pst1 86
Figure 4-2 PBAV-MNO-Map with restriction binding sites. The indicated restriction binding sites have been used to construct the MNO immunity proteins. 87
Figure 4-3: A reverse primer with an initiator codon (ATG) downstream. Figure shows the complex of the multiple cloning site, promoter and His-tag downstream of the ribosomal binding site. 88
Figure 4-4: The structure of newly synthesized plasmid- pBAV1K-T5-gfp expression vector. 89
Figure 4-5: The diagram showing the final construct and the location of the putative immunity gene blpMNO in the blp locus. 92
Figure 4-6: Sequence of the mutated fragment. 93
Figure 4-7: The rectified amino acid sequence of the MNO immunity protein. 94
Figure 4-8: Assay for the MNO immunity protein expression using immunofluorescence staining. 95
Figure 5-1: Inhibition assay on direct co-cultures of the Blp bacteriocin producer TIGR4 WT strain and the target S. parasanguinis cells in BHI media. 105

Figure 5-2: Box and whisker plots of per-base quality statistics generated by the FastQC tool (Andrews, 2010) for DNA extracts of S. parasanguinis across a random sample of 1,754,498.67 mean reads. 110
Figure 5-3: Nx curve of contig length (kbp) against x (%) of the assembly cover. 116
Figure 5-4: The distribution of the number of bases (y-axis) versus coverage values 117
Figure 6-1: Inhibition assay on co-culture of producer TIGR4 WT strain and the different *pneumococci* strains 130
Figure 6-2: Co-culture of TIGR4 strain and Streptococci species in metal ions depleted media. 131
Figure 6-3: Co-culture of TIGR4 and closely related streptococcus species in metal ion-depleted BHI medium supplemented with $CaCl_2$ 100μM 133
Figure 6-4: Co-culture of TIGR4 and closely related streptococcus species in metal ion-depleted BHI medium supplemented with $CaCl_2$ 200μM 134
Figure 6-5: Co-culture of TIGR4 and closely related streptococcus species in metal ion-depleted BHI medium supplemented with $CaCl_2$ 500μM 135
Figure 6-7: Co-culture of TIGR4 and closely related streptococcus species in in metal ion-depleted BHI medium supplemented with $MgCl_2$ 100μM 137
Figure 6-8: Co-culture of TIGR4 and closely related streptococcus species in in metal ion-depleted BHI medium supplemented with $MgCl_2$ 200μM **.** 138
Figure 6-9: Co-culture of TIGR4 and closely related streptococcus species in in metal ion-depleted BHI medium supplemented with $MgCl_2$ 300μM. 139
Figure 6-10: Co-culture of TIGR4 and closely related streptococcus species in in metal ion-depleted BHI medium supplemented with $MgCl_2$ 500μM. 140
Figure 6-11: Co-culture of TIGR4 and closely related streptococcus species in in metal ion-depleted BHI medium supplemented with $MnSO_4$ 2μM. 141
Figure 6-12: Co-culture of TIGR4 and closely related streptococcus species in in metal ion-depleted BHI medium supplemented with $MnSO_4$ 2μM 142
Figure 6-13: Co-culture of TIGR4 and closely related streptococcus species in in metal ion-depleted BHI medium supplemented with $MnSO_4$ 10μM 143
Figure 6-14: Co-culture of TIGR4 and closely related streptococcus species in metal ion-depleted BHI medium supplemented with $MnSO_4$ 50μM 144
Figure 6-15: Co-culture of TIGR4 and closely related streptococcus species in metal ion-depleted BHI medium supplemented $FeCl_2$ 2μM. 145
Figure 6-16: Co-culture of TIGR4 and closely related streptococcus species in metal ion-depleted BHI medium supplemented $FeCl_2$ 5μM. 146
Figure 6-17: Co-culture of TIGR4 and closely related streptococcus species in metal ion-depleted BHI medium supplemented $FeCl_2$ 10μM. 147

Figure 6-18: Co-culture of TIGR4 and closely related streptococcus species in metal ion-depleted BHI medium supplemented $FeCl_2$ 50μM. 148

List of Tables

Table 1.1: Different bacteriocin classes of Gram-positive bacteria (Zimina et al., 2020). 23
Table 2.1: Streptococci strains used in this research study. 45
Table 2.2: List of plasmids. 46
Table 2.3: Conventional PCR primers used in this research study. 57
Table 3.1: Reporters for five BlpC types. 67
Table 3.2: Streptococcal strains tested by overlay assay with the addition of 50 µl of X-gal (40 mg/ml). 67
Table 3.3: Results of overlay assays. 69
Table 5.2: Yield and purity of DNA products extracted from pure colonies of S. Parasanguinis from co-cultures with TIGR4 WT strain and WT S. parasanguinis as the reference strain. 106
Table 5.3: Read Statistics: Forward (F) reads and reverse (R) reads of the paired ends of the DNA samples processed by HTQC 108
Table 5.4: QUAST statistics of the number of genome mis-assemblies without a reference genome 115
Table 5.5: The BBMap output statistics for the short-read alignment. 118

Acknowledgement

I am truly grateful for the support, guidance, and encouragement provided by my supervisor, Professor Tom Evans, throughout my PhD journey. Their expertise and insights have been essential in shaping the direction and quality of my research. I would also like to thank the members of my PhD committee, Dr Lorna Lancaster and Professor Andrew Smith for their valuable feedback, constructive criticism, and valuable suggestions, which have greatly improved the quality of my research.

In addition, I am thankful for the collaborative and supportive research environment created by my colleagues in the faculty of medical veterinary and life sciences. I especially want to acknowledge Dr Lorna Lancaster and Professor Andrew Smith for their valuable discussions and suggestions.

I am grateful to my family and friends for their unwavering support and encouragement. Without their love and encouragement, it would not have been possible for me to complete this journey.

Lastly, I want to thank the funding agency, King Abdulaziz University, for their financial support, which allowed me to focus on my research full-time. I am grateful to everyone who has been a part of my PhD journey and played a role in helping me reach this point.

Abstract

The present study aimed to delineate the functional and structural attributes of the bacteriocin (Blp) locus within the genome of the *Streptococcus pneumoniae* TIGR4 strain, with a specific focus on comprehending the underlying mechanism of action. Prior research had summarised that TIGR4 lacked the capacity for bacteriocin production, despite harbouring a complete Blp locus. In this study, a cohort of co-colonizing bacterial isolates was analysed to evaluate the impact of the Blp locus on bacteriocin and immunity protein synthesis, with the objective of determining whether competitive phenotypes of bacteriocin secretion exert an influence on the dynamics of co-colonization. To this end, a battery of techniques such as overlay assays and a novel inhibition assay were employed. The study results demonstrate that TIGR4 is capable of producing functional bacteriocins and identified the Blp locus as the key determinant of both bacteriocin and immunity protein biosynthesis. Additionally, the study observed that the invasive TIGR4 serotype displayed potent antibacterial activity against *Streptococcus parasanguinis. Streptococcus parasanguinis* has developed resistance to TIGR4-produced bacteriocin after two hours of incubation and next generation sequencing was made to assess the development of mutation or horizontal gene transfer. However, there was no mutation or horizontal gene transfer detected by next generation sequencing. Furthermore, the research examined the role of metal ion transport proteins in modulating bacteriocin activity, as well as assessing the effects of ion deprivation on bacteriocin activity, and the impact of pH and quorum sensing on bacteriocin expression. The present study confirmed that two divalent metal ions, especially Mn^{2+} and Fe^{2+}, are essential for not only the growth of WT TIGR4, but also the bacteriocin-mediated killing of TIGR4 blp KO. The rest of divalent metal ions tested including Ca^{2+} and Mg^{2+} did not support the growth and inhibitory activity of WT TIGR4. Finally, a pneumococcal expression vector was engineered to express a putative immunity

protein with a His-tag, which was then introduced into TIGR4 for assessment of resistance to TIGR4-derived bacteriocins.

List of Abbreviations

ABC	ATP-binding cassette transporters
AMP	Adenosine monophosphate
AUC	Area Under the Curve
BAP	Blood agar plate
BBMap	Brian Bushell's Map
BgaA	Galactosidase A
BHI	Brain heart infusion
BHI	Brain heart infusion
BIR	Bacteriocin/immunity region
BLP	Bacteriocin like peptide
CbpA	Choline-binding protein A
CbpE	Choline-binding protein E
CbpL	Choline-binding protein L
CPS	Capsular polysaccharide
CRM197	Non-toxic mutant of diphtheria toxin
CSP	Competence stimulating peptide
dsDNA	Double-stranded DNA
DSO	Both single-stranded origin
Eno	Enolase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase (
GTAs	Gene transfer agents
HGT	Horizontal gene transfer
HTQC	High-Throughput Quality Control
IgA1	Immunoglobulin A1
IMAC	Immobilized Metal Affinity Chromatography
IPD	Invasive pneumococcal disease
KO	Knock-Out
LAB	Lactic acid bacteria
LB	Luria-Bertani

LDH	Lactate dehydrogenase
NanA	Neuraminidase A
NEB	New England Biolabs
OD	Optical density
OP	Optochin
ORF	Oral Reading Fluency
PAFR	Platelet-activating factor receptor
PCA	Pneumococcal carbonic anhydrase
PCR	Polymerase chain reactions
PCV13	13-valent pneumococcal conjugate vaccine
PCV15	15-valent pneumococcal conjugate vaccine
PCV20	20-valent pneumococcal conjugate vaccine
PHiD-CV	Pneumococcal non-typeable H. influenza protein D conjugate vaccine
PHRED	Phred Quality Score
Pht	Pneumococcal histidine triad protein
PPSV23	23-valent pneumococcal polysaccharide vaccine
Prokka	Prokka: rapid prokaryotic genome annotation
PsaA	Pneumococcal surface adhesin A
PspA	Pneumococcal surface protein A
QSP	Quorum-sensing peptide
QUAST	QUality ASsessment Tool
rMLST	Ribosomal multi-locus sequence typing
ROS	Reactive oxygen species
SJ	Sweet Janus
SNP	Single nucleotide polymorphism
SOB	Super Optimal Broth
SOD	Superoxide dismutase
Spbhp-37	S. pneumoniae haem-binding protein 37
ssDNA	Single-stranded DNA
SSO	Double-stranded origin
THY	Yeast extract media
TIGR4	Streptococcus pneumoniae virulent serotype 4

WT	WILD TYPE
WT	Wildtype
zmpA	Zinc metalloprotease A

CHAPTER 1: Introduction and Literature Overview

1.1 Bacteriology and Epidemiology of *S. pneumoniae*

Streptococcus pneumoniae or pneumococcus is a Gram-positive, non-spore-forming, catalase-negative, and nonmotile coccus or spherical bacteria belonging to the genus *Streptococcus*. This pneumococcus grows in pairs (as diplococci), in groups of four (tetrads), or in chains. They commonly occur in pairs or chains, with the diameter of individual cells being about 0.5-1.5 µm (Zhou and Li, 2015). This organism is frequently found as part of the normal colonising organism in the human nasopharyngeal tract. *S. pneumoniae is* an important pathogen and this is evident by the fact that the World Health Organisation has included it among the 12 priority pathogens (Weiser et al., 2018). The isolates of *S. pneumoniae* are encapsulated with polysaccharide structures (capsular polysaccharide). This capsular polysaccharide (CPS) is an extracellular layer of polysaccharides that surrounds the bacterium that confers protection from harm and phagocytosis by immune cells of hosts (Engholm *et al.*, 2017). At least 95 *S. pneumoniae* serotypes have been identified based on their structurally diverse CPS. Pneumococcus serotypes are different strains of pneumococci that can be differentiated based on the uniqueness of capsular structures. There are more than 95 known serotypes (Zafar *et al.*, 2017)(Ganaie et al., 2020).

The cell wall of *S. pneumoniae* is complex and has various functions that include maintaining the shape of the bacteria and participating in growth and cell division. The bacterial cell wall also plays an important role in the effective interaction of the bacteria with the surrounding environment (Vollmer et al., 2019). Figure 1.1 shows cell envelope of Gram-positive bacteria consistent with *S. pneumoniae*. The *S. pneumoniae* cell wall is composed of capsular polysaccharides, cell surface proteins, peptidoglycan, and wall teichoic acid (Garcia-Bustos et al., 1987; Bui et al., 2012). The surface proteins play an important role in adherence

of pneumococcal to the host cell in a lectin-like approach (Tuomanen, 1997). The various serotypes of *S. pneumoniae* have varying structures of polysaccharide capsule and variation in the surface proteins, which offer the bacteria versatility ensuring successful colonisation of human nasopharynx and eversion of the immune response (Bui et al., 2012).

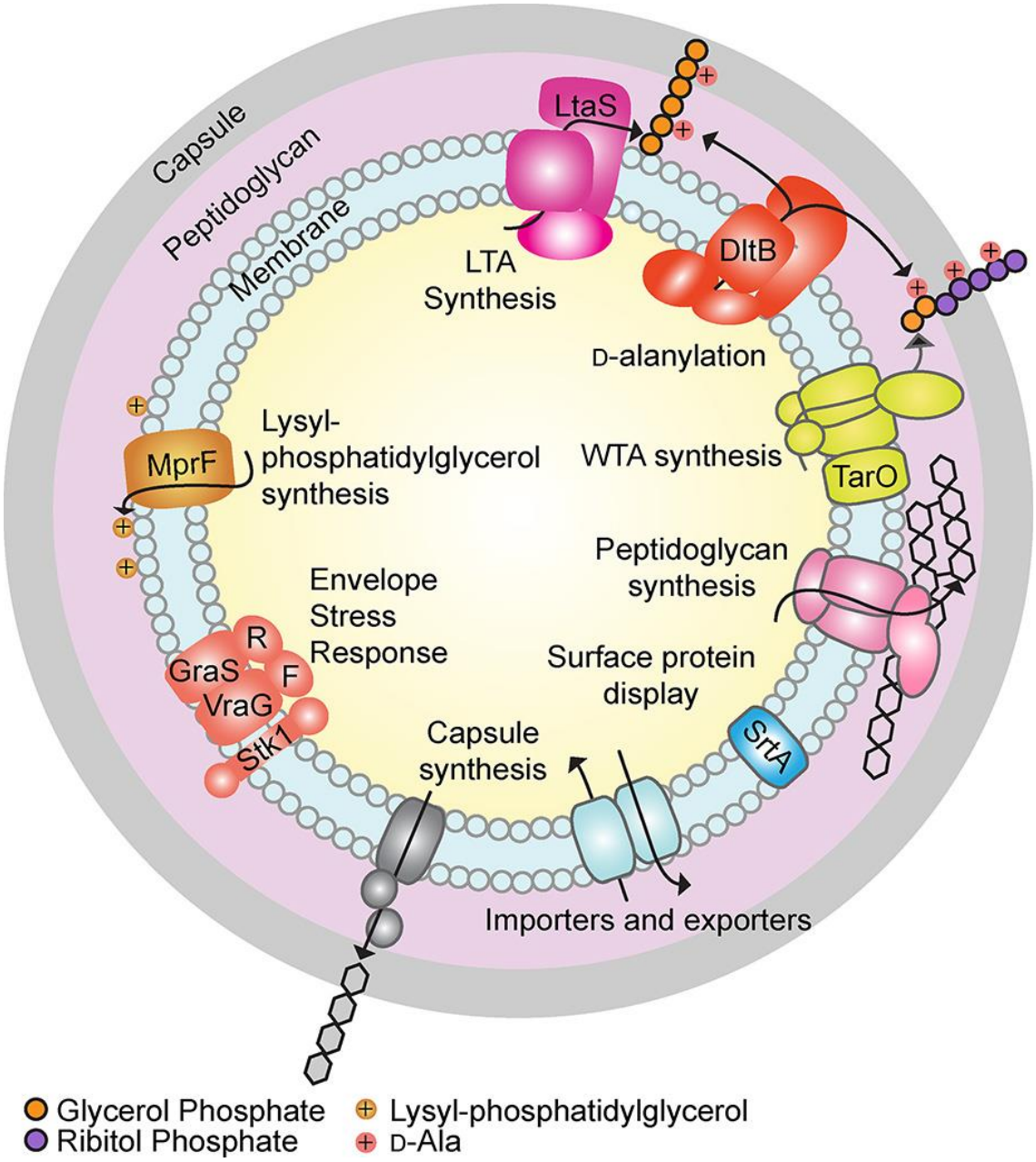

Figure 1-1: The cell envelope Gram-positive bacteria. the cell envelope consists three layers. Starting with the outermost layer is the capsule, peptidoglycan and finally the cell membrane. The peptidoglycan layer which forms a protective shell is 30–100 nm thick (Rajagopal & Walker, 2017).

Peptidoglycan, also called murein, is a mesh-like scaffold around the bacterial cytoplasmic membrane forming the protective part of the Gram-positive bacteria's cell walls. It is a vast polymer of cross-linked chains of identical glycan strands (peptidoglycan monomers) that are further cross-linked by short peptide units (Vollmer, 2015). The peptidoglycan layer of the cell wall is composed of 50 different muropeptides. Effective cross-linking within the peptidoglycan layer is achieved by the stem peptides with glutamate residues that are amidated. Strains of *S. pneumoniae* that are resistant to beta-lactams have dipeptides (L–Ser-L-Ala or L-Ala-L-Ala dipeptides) that are bonded to ε-amino group of L-Lys and therefore, resistant antibiotic lysis action (Vollmer et al., 2019). The presence of N-acetylglucosamine residues that have undergone modification through the removal of an acetyl group and N-acetylmuramic acid residues that have been modified through O-acetylation in the cell wall. This modification makes the *S. pneumoniae's* cell wall resistant to lysozyme action of beta-lactamase antibiotics (Bui et al., 2012). The presence of repeating units of teichoic acid and lipoteichoic acid residues that contain phosphocholine residues anchoring choline-binding proteins make the cell wall of pneumococci unique (Vollmer et al., 2019).

The wall teichoic acid in *S. pneumoniae* is unique compared to those of other Gram-positive bacteria (Bui et al., 2012). Unlike in other bacteria where the membrane anchored lipoteichoic acid has different repeat units from that of the wall teichoic acid, both are similar in *S. pneumoniae*. The *S. pneumoniae* wall teichoic acid also contains 2-acetamido-4-amino-2, 4, 6-trideoxy-galactose, which is a rare amino sugar. The pneumococci wall teichoic acid also contains phosphocholine residues not found in other bacteria (Bui et al., 2012). *S. pneumoniae* can cause invasive diseases such as pneumococcal pneumonia that results in bacteraemia and meningitis or non-invasive pneumococcal diseases such as pneumonia that is localised in the lungs and sinusitis (Tin Htar et al., 2017; Marquart, 2021).

The variation in the distribution of the different serotypes across various regions is determined by the selective pressure of antibiotics and the use of vaccines (Chen et al., 2018; Lewnard & Hanage, 2019). Evidence suggests that vaccine non-responsive serotypes take up the niches vacated by the pneumococcal conjugate vaccine (Lewnard & Hanage, 2019). It has been observed in developed countries that have previously implemented vaccination programs that the gains registered with the reduction in the carriage of vaccine-targeted serotypes were nullified by the increase in non-vaccine serotypes (Hanage et al., 2010; Flasche et al., 2011; Weinberger et al., 2011; Feikin et al., 2013). The implementation of the 13-valent pneumococcal conjugate vaccine resulted in a reduction in the incidences of serotypes 1, 6A, and 7F (Weinberger et al., 2011; Lewnard & Hanage, 2019). However, recent UK data show an increase in all invasive pneumococcal diseases among the adults and the elderly and the increasing serotype replacement among the children (Lewnard & Hanage, 2019). In the UK and US, pneumococcal serotypes 8, 9N, 12F, and 15A have been noted to cause increased cases of invasive pneumococcal disease (IPD) (Yildirim et al., 2017; Ladhani et al., 2018). Serotypes 8 and 12F frequently cause moderately severe IPD in younger or healthier individuals and not likely to result in fatal outcomes. Serotype 15A frequently cause fatal cases of IPD in older adults with comorbidities (Amin-Chowdhury et al., 2020). Based on the analysis of national invasive pneumococcal disease surveillance data for 2016 and 2017, Ladhani et al. (2018) observed that about 40 per cent of invasive pneumococcal diseases in England and Wales were attributed to serotypes 8,12F, and 9N.

The emergence and distribution of pneumococcal serotypes that are not sensitive to the vaccines currently on the market continue to be reported (Kavalari et al., 2019). Serotype 24F is an example of a pneumococcal serotype that is not sensitive to treatment using penicillin and cannot be controlled using pneumococcal conjugate vaccines (Muñoz-Almagro et al., 2011; Balsells et al., 2017; Camilli et al., 2017). Serotype 24F is also associated with

erythromycin and tetracycline resistance (Janoir et al., 2016). Kavalari et al. (2019) analysed the invasive pneumococcal infection surveillance data in Denmark between 1999 and 2016 and they noted an increase in invasive pneumococcal disease cases attributed to serotype 24F. According to Chen et al. (2018), the most prevalent pneumococcal serotype in China is serotype 19F.

The mean age of *S. pneumoniae* acquisition is higher in developed countries (six months) compared to developing countries (three months) (Arguedas et al., 2020). The reported variation in the mean age of acquisition between developed countries and the developing ones could be attributed to variations in the transmission conditions and environmental factors such as crowding (Arguedas et al., 2020). The differences in immunisation approaches and coverage also contribute to the variation in the reported mean age of pneumococcal acquisition among children (Arguedas et al., 2020). Kartasasmita et al. (2020) reported pneumococcal nasopharyngeal carriage in up to 55 % of healthy Indonesian children below the age of 5 years. The researchers further observed from serotype analysis that up to 60 % of the pneumococcal nasopharyngeal carriage isolates are sensitive to the 13-valent pneumococcal conjugate vaccine (PCV13). There are three PVC (PCV13, PCV15, and PCV20) and one 23-valent pneumococcal polysaccharide vaccine (PPSV23) have been developed to protect children and adults against pneumococcal infection (Daniels et al., 2016)(CDC, 2023).

De Cellès et al. (2019) reported that the distribution of diseases caused by *S. pneumoniae* varies based on climate changes and age with more cases being reported among the elderly in the population. Evidence showed that the variation in temperature, humidity, sunshine duration and UV radiation influence the variability in invasive pneumococcal disease. De Cellès et al. (2019) and other researchers (Walter et al., 2010) have shown that the transmission of pneumococcal disease is higher during dry and cool seasons. According

to Torres et al. (2018), the burden of community-acquired pneumonia among adults in Europe is as high as 7000 per 100,000 with the burden of hospitalized community-acquired pneumonia increasing with the increasing age. In the United Kingdom, *S. pneumoniae* is the most prevalent bacteria in community-acquired pneumonia, accounting for almost 50% of the cases, with a mortality rate of at least 20% in patients with a systemic infection, such as pneumococcal septicaemia (Brown, 2012; Cheng *et al.*, 2018). The mortality is even higher in developing countries due to increased risk of pneumococcal septicaemia, where it accounts for 25% of all unnecessary deaths in children below the age of 5 and more than 1.2 million deaths of infants every year (World Health Organization, 2013). There are several virulence determinants of infectious strains of *S. pneumoniae*. Therefore, the present study aims to review factors that enable *S. pneumoniae* colonization and infection in human hosts.

1.2 Literature Review

1.2.1 Colonisation and Invasion

S. pneumoniae is a common coloniser of the nasopharynx. The degree of carriage is modified by numerous factors including ethnicity, and socioeconomic conditions. Carriage of *S. pneumoniae* is common in children, especially in resource-limited settings (Bogaert *et al.*, 2011)(Daningrat et al., 2022). Initial colonisation with *S. pneumoniae* can appear as early as one month of age and the rate of carriage reaches up to 55% at three years of age due to inadequate immune capacity to clear or supress *S. pneumoniae* colonisation (Bogaert *et al.*, 2011). The carriage is about 5-10% in adults, because of increased immunocompetency aiding clearance and suppression of *S. pneumoniae* colonisation (Daningrat et al., 2022). In developing countries, about 40% of healthy populations are carriers of *S. pneumoniae* and with extremely higher rates of up to 95% in infants and children. The duration of carriage is inversely related to age, ranging from five days to months. Colonisation with more than two serotypes is also most commonly observed in recent years (Högberg *et al.*, 2007). The CPS

has been identified as one of the primary virulence factors of pneumococcus that determines its colonization (Weinberger *et al.*, 2009).

S. pneumoniae is mainly located in the epithelial region covered by the mucus layer (Weiser et al., 2018). The shedding of pneumococci from the colonised host involves the expression of mucin glycoproteins. Based on studies that focused on co-infection with influenza A virus, it was noted that the shedding of the pneumococci is associated with the degree of upper respiratory tract inflammation resulting from the viral infection (Weiser et al., 2018). The inflammation caused by a deficiency of toll-like receptors resulting from the increasing viral load increases the transmission of the bacteria. Pneumolysin (pore-forming toxin) also causes inflammation, stimulating the secretion of mucus and increasing the shedding of the bacteria, which indicates the link between pneumococcal virulence and transmission (Lipsitch and Moxon, 1997; Matthias et al., 2008). The presence of agglutination antibodies such as immunoglobulin G reduces shedding; however, pneumococcal proteases can cleave such antibodies further increasing the shedding (Weiser et al., 2018). Rodrigues et al. (2013) observed that among children, the volume of mucus secretion is associated with the amount of *S. pneumoniae*.

Once shed from the upper respiratory tract the pneumococci can infect a healthy person directly or through contact with contaminated surfaces (Zafar et al., 2017; Weiser et al., 2018). The transmission of pneumococci to healthy individuals can occur through close contact and aerosols (Marquart, 2021). *S. pneumoniae* can survive outside the host for days if there are ambient nutrient sufficient conditions (Verhagen et al., 2014). Pneumolysin increases the survival of the bacteria in poor nutrient conditions (Zafar et al., 2017). The expression of the capsule avails glycan reserves to the bacteria, increasing the survival of pneumococcal in poor nutrient conditions (Hamaguchi et al., 2018).

Various factors influence the acquisition of the new host by *S. pneumoniae* with one of the conditions being the pre-existing colonisation (Kono et al., 2016). The target host organisms with pre-existing pneumococcal colonisation are less likely to be acquired as new hosts (Kono et al., 2016). The acquisition of a new host can also be blocked by passive immunisation (Roche et al., 2015). The bacteria also use pneumococcal zinc metalloprotease ZmpA to overcome agglutinating action of IgA1 by cleaving the immunoglobulin at the hinge region (Roche et al., 2015). However, the target hosts that have been immunised with pneumococcal conjugate vaccines may not be acquired because of PCV induced IgG, which is less susceptible to cleavage by ZmpA (Weiser et al., 2018). Pennington et al. (2016) noted that the levels of capsular polysaccharide specific memory B cells determine the acquisition.

The colonisation of the host surfaces by *S. pneumoniae* occurs in a stepwise manner; the first step is establishing contact with the epithelial receptors, after which the bacteria interact with the complement system then it degrades the mucus; initiates metal binding and causes impairment of neutrophil action (Weiser et al., 2018). An important step in pneumococcal pathogenesis is the attachment of the bacteria to the host cell. The virulence factors that help in the attachment step include pneumococcal surface adhesin A (PsaA), which attaches to the E-cadherin of the host (Anderton et al., 2007; Marquart, 2021). The second factor needed for attachment is the choline-binding protein A/pneumococcal surface protein C/*S. pneumoniae* secretory IgA binding protein (CbpA/PspC/SpsA), which attaches to sialic acid, lacto-N-neotetraose, the polymeric immunoglobulin (Ig) receptor, and vitronectin (Voss et al., 2013; Marquart, 2021).

However, for the bacteria to attach to the host cell, there is a need to evade the natural barriers that include respiratory mucus and lysozyme (Nelson et al., 2007). To evade the mucus entrapment, pneumococci use the negative charge of the CPS to repulse sialic acid in the mucus, avoiding entrapment (Mook-Kanamori et al., 2011). *S. pneumoniae* also produces

exoglycosidases that deglycosylate the glycoconjugates in the mucus, therefore, interfering with its viscosity and limiting entrapment. Some of the enzymes used to degrade mucus include Neuraminidase A (NanA), β-galactosidase A (BgaA) and β-N-acetylglucosaminidase (StrH). The degradation of mucus results in reduced mucociliary clearance (Weiser et al., 2018). Pneumolysin produced as a result of autolysin induction also enables pneumococci to avoid being removed by mucociliary clearence from the airways in the respiratory system. Pneumolysin reduces ciliary beating by damaging the epithelial (Weiser et al., 2018). To evade the degrading effects of lysozyme on the peptidoglycan layer, pneumococci produce peptidoglycan N-acetylglucosamine-deacetylase A and O-acetyltransferase enzymes that modify the layer and make it resistant to cleavage (Mook-Kanamori et al., 2011). The pneumococcal adherence to airway epithelia is facilitated by platelet-activating factor receptor (PAFR) expressed on the surface of airway epithelial cells. A pharmacological blockade of PAFR has been shown to decrease the bacterial binding activity to this receptor *in vitro* (Bhalla et al., 2020). PAFR receptors exhibit constitutive activity or inverse agonism, meaning that their blockade can stop pneumococcal adherence to and colonisation of airway epithelia (Weiser et al., 2018). The adherence is also aided by peptidyl-prolyl isomerase SlrA (Hermans et al., 2006). Upon attachment of the host cell, the next step would be the establishment of pneumococcus in various locations such as the lungs, blood, or the central nervous system (Marquart, 2021).

The approach used by various pneumococci bacteria in establishment varies based on the bacterial gene expression regulation, susceptibility of the host and how the bacteria interact with the host components (Marquart, 2021). Pneumococcal pathogenesis resulting in pneumococcal pneumonia is characterised by the cleavage of sialic acid from the glycoproteins receptors of the host cell by pneumococcal neuraminidase. The cleavage of sialic acid facilitates the attachment of the bacteria to the host airway epithelial cells (Tong et

al., 1999). Pneumolysin, a cholesterol-dependent cytolysin produced by pneumococci elicit pro-inflammatory cytokine responses and takes part in pore formation in the cell membranes (Feldman et al., 1991). The migration of *S. pneumoniae* to the lungs and blood is aided by choline-binding protein L (CbpL) (Gutiérrez-Fernández et al., 2016). Following the breach of mucosal barriers, pneumococci can cause local and disseminated infections (Wholey et al., 2019). Pneumococci can then spread to the blood causing septicaemia (Marquart, 2021). Pneumolysin enables the bacteria to escape the host immune system by promoting the uptake of the bacteria and escape from the lysosome (Marquart, 2021). The virulence factors that facilitate pneumococci to acquire metal ions from the host cell include the piuA, piaA and pitA, which are used for iron acquisition (Subramanian et al., 2019). Pneumococci also use psaA in the acquisition of magnesium ions and adcA and adcAII for zinc ions binding (Subramanian et al., 2019).

Once in the circulation, pneumococcal surface protein A (PspA) aids the survival of the bacteria by short-circuiting bacterial opsonization (Marquart, 2021). The capsule also helps the bacteria avoid phagocytosis by masking the surface antigens (Brown et al., 1983). Other pneumococcal proteins such as choline-binding protein A (CbpA), enolase (Eno) and pneumococcal histidine triad protein (Pht) block the complement system (Weiser et al., 2018). The choline-binding protein E (CbpE) causes impairment of neutrophil action through the degradation of the platelet-activating factor (Weiser et al., 2018). Successful colonization of *S. pneumoniae* is also determined by its interaction with the microbiota in the nasopharynx (Weiser et al., 2018). The larger the diversity of microbiota in the nasopharynx the increased the acquisition of *S. pneumoniae* (Cremers et al., 2014). Increased inflammation in the upper respiratory tract increases the likelihood of colonisation by *S. pneumoniae* (Weiser et al., 2018).

1.2.2 Pathogenesis and Virulence of *S. pneumoniae*

The features that aid pneumococci pathogenicity is the high carriage rates, the shift from commensal to pathogenic organisms, and the ability to carry out genetic adaptation (Weiser et al., 2018). Isolates of pathogenic pneumococci have a thick encapsulation indicating that thick CPS is among its virulence factors. In certain infectious strains, the amount of CPS (thick layer of CPS) was directly linked to virulence (Engholm *et al.*, 2017; Kadioglu *et al.*, 2008). As described by Kadioglu et al. (2008), the capsule of pneumococcus can interact with the immune arsenal of the host in numerous ways. For example, it evades phagocytosis by reducing the degree of complement activation on its cell wall surface, disturbing the interaction between complement and its receptors on phagocytic cells, and limiting its level of interaction with phagocytes by remaining positively charged at physiological pH. Characterisation of the co-colonisation of different strains at the level of serotype and genotype has allowed investigation of a positive association between specific capsular types and genotypes (Hathaway *et al.*, 2012; Li *et al.*, 2013). Pneumococcal causes meningitis upon passing the blood-brain barrier (Subramanian et al., 2019). The bacteria are able to evade microvascular endothelial cells in the barrier by binding endothelial receptors (platelet endothelial cell adhesion molecule 1 and polymeric immunoglobulin receptor) using pneumococcal pilus-1 adhesin RrgA and PspC (Iovino et al., 2014; Iovino et al., 2016). Evidence indicates that transparent pneumococcal variants unlike the opaque variants, are more likely to cross brain microvascular endothelial cells (Ring et al., 1998).

Survival of pneumococci in the nasopharynx is an important step in bacterial pathogenesis (Wholey et al., 2019). The presence of other respiratory bacteria such as *Mycoplasma pneumoniae*, *Staphylococcus aureus, Haemophilus influenzae*, and *Chlamydophila pneumoniae* in the nasopharynx can affect the colonisation of *S. pneumococciae* and invasiveness. Bacteriocins, are ribosomal-synthesized antimicrobial peptides produced by

bacteria that inhibit or kill other related or unrelated competing species (Perez *et al.*, 2014). Using bacteriocins, pneumococci eliminate competition and therefore acquires nutrients and space for proliferation (Wholey et al., 2019). A previous study revealed that adherence of pneumococci was prevented *in vitro* by bacteriocins produced by *Streptococcus salivarius* isolated from nasal and/or pharyngeal swabs of healthy children (Santagati *et al.*, 2012). In contrast, the *in vivo* colonisation (cell concentration) of pneumococcus in the nasopharynx was shown to correlate positively with *H. influenzae* colonisation, but negatively with *S. aureus* (Chien et al., 2013). These pieces of evidence from the literature suggest that *S. pneumoniae* suppresses other closely related species by producing genus-specific bacteriocins.

There is limited research on the immunomodulatory properties of bacteriocins (Brand et al., 2013). Some of the limited information indicates that some bacteriocins such as nisin A and Z could be toxic to human lymphocytes and neutrophils (Bedge et al., 2011). An early study carried out by De Pablo et al. (1999) reported an increase in CD4 and CD8 T lymphocytes as a result of the administration of class I bacteriocin called nisin. De Pablo et al. (1999) also observed a reduction in B lymphocytes. Following prolonged administration of nisin, De Pablo et al. (1999) also observed increased phagocytic action of peritoneal cells. The study carried out by Villamil et al. (2003) showed that the administration of nisin in fish causes immunostimulatory effects on the kidney macrophages.

Evidence suggested that the virulence factors found in *S. pneumoniae* can also be found in genetically related strains of bacteria such as *Streptococcus mitis* and *Streptococcus pseudopneumoniae* (Kilian & Tettelin, 2019). Pneumococcus and S. mitis belong to the Mitis streptococci group. The similarity between strains could be a result of the species sharing an immediate ancestor (Kilian et al., 2008; Kilian et al., 2014.)**.** Due to common ancestry, strains of *S. pneumoniae* and *S. mitis* share virulence factors such as pneumolysin and autolysin

(Kilian & Tettelin, 2019). The two species also have neuraminidases A and B. Strains of *S. mitis* also have homologues of choline-binding proteins found in *S. pneumoniae*. Some strains of *Streptococcus* spp. share similar bacteriocin features to the *S. pneumoniae* strain. According to Kilian and Tettelin (2019), strains of *S. mitis* and *S. pseudopneumoniae* have Blp bacteriocin cassettes similar to that of the *S. pneumoniae* strains. Kilian and Tettelin (2019) noted that the three related organisms have bacteriocin cassettes located in the same context within the genome. Some strains of *S. mitis* and *S. pseudopneumoniae* have also been observed to have both ABC transporters *blpB* and *blpA* that are intact.

The *comA* and *comB* gene cassettes which play a critical role in *S. pneumoniae natural* competence for genetic transformation have also been observed in strains of *S. pseudopneumoniae* but not in *S. mitis* (Kilian & Tettelin, 2019)(Gagne et al., 2013). A competent *S. pneumoniae* cell expresses CibABC, a two-peptide bacteriocin, in response to increased accumulation of secreted peptide pheromone. This is one of the bacterium quorum sensing strategies that enable them to target *S. pneumoniae* cells lacking functional Cibc expression (non-competent cells) for destruction via lysis (Shanker & Federle, 2017). Evidence also indicates that strains of *S. pseudopneumoniae* have the cibABC locus encoding the immunity protein CibC (Kilian & Tettelin, 2019). Competent *S. pneumoniae* cell uses the CibAB component to induce the lysis of the closely related non-competent cells (Gómez-Mejia et al., 2018). Kilian and Tettelin (2019), also reported that strains of *S. mitis* have *cibA* and *cibB*. However, (Lee et al., 2019) did not find the *cibA* and *cibB* in strains of *S. mitis*.

In addition to the highlighted similarities between *S. pneumoniae* and *S. mitis*, it should be noted that both organisms are important pathogens responsible for various diseases among humans (Kilian & Tettelin, 2019). Some strains of *S. mitis* can cause various invasive diseases, especially among immunocompromised individuals and are one of the major oral streptococci that cause bacteraemia (Mitchell, 2011; Alves et al., 2019). As is the case with

S. pneumoniae, *S. mitis* also colonises children's oropharynx (Mitchell, 2011). S. mitis is the most predominant commensal bacteria in multiple oropharyngeal sites, including mucosal and dental surfaces, which persists throughout life (Engen et al., 2014; Harth-Chu et al., 2019). However, S. mitis can cause infectious complications including infective endocarditis, bacteraemia and septicaemia (Sadowy & Hryniewicz, 2020).

1.2.3 Diagnosis and Treatment

Disease caused by pneumococcus can be diagnosed by culturing nasal/pharyngeal samples from suspected cases. A positively identified pneumococcal infection can be treated with penicillin, macrolide, and other newer classes of antibiotics (Kang et al., 2013). The initial treatment strategies for individuals with community-acquired pneumonia who show no comorbidities and with no evidence of multi-drug resistant *Staphylococcus aureus* or *Pseudomonas aeruginosa* include amoxicillin or doxycycline or a macrolide (Metlay et al., 2019). However, for patients with comorbidities such as diabetes, alcoholism, chronic conditions of the heart and liver or renal diseases, the recommended initial treatment includes combined therapy with amoxicillin/ cephalosporin and macrolide/ doxycycline. Individuals with comorbidities could also be treated with monotherapy using fluoroquinolone (Metlay et al., 2019). For patients with non-severe inpatient pneumonia, beta-lactam and macrolide type antibiotics could be used as the standard regimen. For severe cases of inpatient pneumonia, beta-lactam and fluoroquinolone could be used as a standard regimen (Metlay et al., 2019). Treatment of acute otitis media involves the management of pain and control of infectious processes using antibiotics (Danishyar & Ashurst, 2017). Pain management is achieved through the use of nonsteroidal anti-inflammatory drugs or acetaminophen (Marchisio et al., 2019). First-line antibiotics used in the treatment of suppurative acute otitis media include amoxicillin or second-generation cephalosporin. Azithromycin and clarithromycin are also used among individuals with penicillin allergies (Danishyar & Ashurst, 2017). Amoxicillin-

clavulanate is used if symptoms persist after treatment with first-line antibiotics (Lewnard et al., 2018).

Vaccines are also used a means of preventing pneumococcal infections. The vaccines that are used against pneumococci include the 23-valent pneumococcal polysaccharide-based vaccine (PPV23) and pneumococcal conjugate vaccines (PCVs) (Masomian et al., 2020). The two vaccines include the serotypes (such as serotype 19A) that are responsible for invasive pneumococcal disease (Isturiz et al., 2017). The PPV23 is a polysaccharide vaccine derived from capsular polysaccharide antigens acquired from 23 different pneumococcal serotypes that cause about 90% of invasive infections (Orange et al., 2012). The PPV23 are given to the elderly (above 65 years) and individuals aged between two and 64 years with comorbidities such as diabetes (Masomian et al., 2020). Evidence suggests that PPV23 help to lower the severity of community-acquired pneumonia but cannot prevent it (Torres et al., 2018; Wijayasri et al., 2019; Masomian et al., 2020). The inability of PPV23 to produce immunological memory has been associated with its ineffectiveness in children. However, among individuals below the age of 75 years, PPV23 has been shown to be effective in preventing invasive pneumococcal disease (Moberley et al., 2013; Masomian et al., 2020).

The PCVs are relatively newer having been introduced in 2000. There are various PCVs in the market today with one of them being the 13-valent pneumococcal conjugate vaccine (PCV13), which consists of conjugates of capsular polysaccharide antigens, non-toxic mutant of diphtheria toxin (CRM197) (Masomian et al., 2020). The other PCV is the 10-valent pneumococcal non-typeable *H. influenza* protein D conjugate vaccine (PHiD-CV), which is made up of the following conjugates: (1) pneumococcal polysaccharides and non-typeable *H. influenza* carrier protein D, (2) serotype 18C and tetanus toxoid, and (3) serotype 19F and diphtheria toxoid. Unlike PPV23, PCV13 show the capacity to elicit memory t-cells response and therefore has a greater ability to offer protection against infections (Song et al., 2013).

Evidence indicates that the pneumococcal conjugate vaccine can reduce the risk of pneumonia in children and promotes herd immunity by reducing carriage (Poolman et al., 2013; Olarte et al., 2017). One challenge associated with the pneumococcal conjugate vaccine is that its effectiveness is based on the serotypes used in its development but the inclusion of more serotypes into the formulation is challenging due to the cost and complexity (Poolman et al., 2013).

Pneumococcal conjugate vaccines (PCVs) are given to children in numerous developed countries and some developing countries to boost immune against infection in children (Bogaardt *et al.*, 2015). The CDC recommends PCV13 or PCV15 for children below 5 years or immunocompromised children aged 5-18 years. PCV20 is suitable for people aged 65 years or older or immunocompromised adults aged > 19 years (Centers for Disease Control and Prevention -CDC, 2023). 23-valent pneumococcal polysaccharide vaccine (PPSV23) is the most recent vaccine developed to protect against 80-90% of the pneumococcal capsular serotypes including 8,12F, and 9N among children and adults (Daniels et al., 2016) (CDC, 2023). The administration of PCVs has considerably decreased illness and death in those countries (WHO, 2012). However, modern vaccines only target antigens for 7 or 13 serotypes of pneumococci, and rare serotypes are beginning to replace those targeted by the vaccines. New generation conjugate vaccines include 10 (PCV10) or 13 (PCV13), but these resistant serotypes are being reported each year (Dobay, 2019).

Evidence indicates an upsurge in the prevalence of diseases attributed to the serotypes that are not included in the vaccines, a phenomenon referred to as serotype replacement (Geno et al., 2015; Geno et al., 2017). According to Koelman et al. (2020), the initial decline in the incidences of pneumococcal meningitis as a result of the introduction of conjugate pneumococcal vaccines was temporary. The researchers reported that serotype replacement has led to increased incidences of non-vaccine serotypes, leading to the continued challenge

of pneumococcal meningitis. Isturiz et al. (2019) examined the burden of adult pneumococcal diseases post 13-valent pneumococcal conjugate vaccine (PCV13) and took into consideration the various serotypes. Following the analysis of data obtained from American adults recruited from acute care hospitals, Isturiz et al. (2019) reported that 4.6 % of the patients had PCV13 serotypes, which shows the persistent presence of the disease despite the implementation of the vaccination program. Isturiz et al. (2019) noted that the risk of infective pneumonia was high among patients with chronic obstructive pulmonary disease, diabetes and those who smoked. Wijayasri et al. (2019) also examined the distribution of invasive pneumococcal disease after the implementation of PCV13. Wijayasri et al. (2019) based their research on data collected from invasive pneumococcal disease cases in Ontario Canada. The researchers recorded a significant reduction in the incidence of the disease caused by PCV13 serotypes. However, the researchers reported an increase in the incidence of disease between 2007 and 2017 resulting from 23-valent pneumococcal polysaccharide vaccine (PPV23) serotypes despite the vaccines being available since 1996. Wijayasri et al. (2019) also noted that the incidence of pneumococcal disease due to non-vaccine-preventable serotypes also increased across all age groups. The researchers, therefore, concluded that despite PCV13 having direct and indirect effects on the incidence of pneumococcal diseases the overall rate remained unchanged and invasive pneumococcal disease continues to cause a severe burden on the public health in Canada.

The reported decline in the effectiveness of the existing vaccines coincide with the increased treatment failure. *S. pneumoniae* causes acute respiratory infections such as pneumonia that do not respond to conventional antibacterial remedies (Cardoso et al. 2008). Penicillin-resistant pneumococcal strains that are insensitive to empirical antibiotic therapy have been reported (Cardoso et al. 2008; Morley et al. 2017). Treatment failure in pneumonia is associated with poor clinical outcomes (Cardoso et al. 2008). Treatment failure requires

escalation of treatment for ICU admission (Morley et al., 2017). Musher and Thorner (2014) noted that community-acquired pneumonia continues to be a burden despite the global administration of vaccines against the disease.

The use of antibiotic is also challenged by the widespread resistance of pneumococci to a wide range of antibiotics, recovered globally. For instance, 60% of pneumococci recovered in Asia are multidrug-resistant (Kang et al., 2013). *S. pneumoniae* obtain the capacity to resist antibiotics and evade the host immune system through the remodelling of its genome which is facilitated by the uptake and the incorporation of the DNA from the pneumococcal and related pathogenic organisms (Weiser et al., 2018).The resistance of *S. pneumoniae* to treatment is not a new phenomenon given that multi-drug resistant pneumococcal was reported as early as 1978 (Jacobs et al., 1978).Evidence suggests that the development of antibiotic resistance in strains of *S. pneumoniae* is a result of the uptake of antibiotic resistance genes from the less pathogenic relative *S. mitis* (Mitchell, 2011). Kilian et al. (2014) further noted that the relationship between *S. pneumoniae* and *S. mitis* is characterised by unidirectional manner interspecies transfer of genes from *S. mitis* to *S. pneumoniae*. Kilian et al. (2014) attributed the diverse nature of the *S. pneumoniae* to interspecies transfer of genes between *S. mitis* to *S. pneumoniae*.

The increased number of drug resistance pneumococcal in 1980 coincided with the increase in treatment failures and recurrent diseases caused by the bacteria such as acute otitis media (Kristinsson, 1999).The problem of pneumococcal treatment failure has a long history with most researchers documenting the resistance of the bacteria to penicillin treatment (John, 1994; Blasi et al. 2012; Pumarola et al., 2013).Treatment failure, which is characterized by the persistence of symptoms two to three days after the administration of the required antibiotics is a problem that has been reported in the treatment of pneumococcal diseases such as acute otitis media (Pumarola et al., 2013; Eythorsson et al., 2018).Treatment failure is

associated with increased complications and length of hospitalization and could also increase the risk of death, especially among those with severe community-acquired pneumonia (Dinh et al., 2021).

S. pneumoniae strains that are not susceptible to penicillin are the leading causes of treatment failure (Eythorsson et al., 2018). Zielnik-Jurkiewicz and Bielicka (2015), reported that *S. pneumoniae* was the most frequently isolated pathogen among children with acute otitis media treatment failure. Zielnik-Jurkiewicz and Bielicka (2015) based their observations on a prospective study involving 157 children with acute otitis media between the ages of 6 months and 7 years who were admitted because of an unsuccessful oral antibiotic therapy. Zielnik-Jurkiewicz and Bielicka (2015) further noted that 65.4 % of *S. pneumoniae* strains were not sensitive to penicillin while 67.2 % of the strains were multidrug-resistant. Pumarola et al. (2013) analysed 105 acute otitis media episodes from children aged between 3 to 36 months and they noted that 56 % of pneumococcal isolates had multidrug resistance. For community-acquired pneumonia, the treatment failure rate is between 6 % to 24 % and could be as high as 31 % for severe cases (Dinh et al., 2021). Based on the analysis of data obtained from 1236 patients with community-acquired pneumonia, Ott et al. (2012) observed that 15.9 % of the patients had treatment failure, which prolonged the hospital stay and increased the cost of treatments. Cardoso et al. (2008) analysed data from 240 children between the ages of 3 to 59 months who were hospitalised with severe pneumonia and they reported treatment failure in 21 % of the cases.

To address the challenge of antibiotic treatment failure and growing ineffectiveness of PPV23 and PCV13, there is a need to develop alternative vaccines that boost mucosal and systemic immunity and prevent the colonization of the nasal cavity by the bacteria and hinder the invasive diseases (Masomian et al., 2020). The alternative options include protein-based vaccines developed from conserved sequences of proteins such as pneumococcal surface

protein A (Nagano et al., 2018), pneumococcal surface protein C (Dieudonné-Vatran et al., 2009), pneumolysin (Miyaji et al., 2013) and pneumococcal peptide 27 (Kim et al., 2012). The other alternative vaccines include whole-cell vaccines that express all protein antigens (Masomian et al., 2020).

1.2.4 Antimicrobial Peptides (AMPs)

AMPs are relatively small peptides no longer than 50 amino acids. Their structure can be linear, circular, α-helices or β-sheets. Bacteriocins are antimicrobial peptides that are synthesised in the ribosome of bacteria. AMPs are produced by all known organisms, probably except for viruses (Nissen-Meyer *et al.*, 2009). While AMPs produced by the host cell function to inhibit pathogenic bacteria, AMPs produced by microorganisms (such as bacteriocins) function to inhibit growth activities of similar or closely related rival strains (Simons *et al.*, 2020). AMPS have been shown to be selective against either Gram-positive bacteria, Gram-negative bacteria, or fungi (Brogden, 2005; Mahlapuu *et al.*, 2016).

Strikingly, most AMPs are cationic and is believed that the positive charge present on the AMPs is necessary to attract the regardless of cell wall peptidoglycan content (Gram stain status). Both Gram-positive and Gram-negative bacterial cell surfaces possess net negative electrostatic charge (Wilson et al., 2001). The negatively-charged bacterial cell membranes is useful for onward binding with cationic AMPs (Islam *et al.*, 2012; Steckbeck *et al.*, 2014). On the other hand, one of the amazing characteristics of these molecules is their potency at extremely low concentrations (nM-μM range)(Cotter *et al.*, 2005; Islam et al., 2012). Microbial AMPs include bacteriocin, lantibiotics, and fratricide.

1.2.4.1 Bacteriocins.

Bacteriocins are AMPs that are synthesised in the ribosome of bacteria (Nissen-Meyer et al., 2009). Gratia is credited with the discovery of the first bacteriocin called colicin in 1925 (Gratia and Fredericq, 1946). bacteriocin production is typical in organisms that reside in

poly-microbial communities. bacteriocins are produced by Gram-positive bacteria and Gram-negative bacteria (Drider et al. 2016). For lactic acid bacteria, the genes responsible for the production of bacteriocin are situated on the plasmids or on the chromosomal DNA (Perez et al. 2014; Drider et al. 2016). bacteriocins are encoded by various microbes in the human gut. A study carried out by Drissi et al. (2015) identified structural classification of putative bacteriocins encoded by 317 microbial genomes in the human gut. At least 175 bacteriocins are encoded in firmicutes, 79 in proteobacteria, 34 in bacteroidetes, and 25 in actinobacteria (Drissi et al., 2015). bacteriocins are a diverse group of compounds in terms of size, mode of action, and immunity mechanisms. The competition mediated by bacteriocin production has been shown to alter the composition of the microbiota in environmental communities and on human surfaces (Riley and Wertz, 2002).

1.2.4.1.1 Classification of bacteriocins.

As noted by Chikindas et al. (2018), the classification of bacteriocins can be confusing. Rezaei et al. (2018) in the study that sequenced pneumococci genome to identify antimicrobial peptides observed that the bacteria have greatly diverse bacteriocin clusters. Rezaei et al. (2018) noted that the bacteriocin clusters in pneumococci are also present in other streptococcal species. One classification approach involves the two groups (true bacteriocins and the bacteriocin like inhibitory substances (Manning et al. 2016). A more elaborate classification of bacteriocins is based on the origin, production complexity, and the mechanisms of action (Chikindas et al. 2018). Gradisteanu Pircalabioru et al. (2021) noted that the classification systems for bacteriocins are ever-evolving as new groups are discovered and the complexities of existing ones are increasingly being understood. The features that are currently used in the classification of bacteriocins include their origin, physical and chemical properties, amino acid composition and their intrinsic function.

One group of bacteriocins is the lantibiotics, which are also known as class I bacteriocins. Lantibiotics are produced by Gram-positive bacteria with activity primarily against other Gram-positive strains (Islam et al., 2012). The class I bacteriocins are made up of peptides with a molecular weight of not more than 5 kDa. The group contains heat resistant peptides consisting of between 19 and 50 amino acids, which confer proteolytic action on the membrane of the target organism forming pores and intracellular rings, which result in cell lysis (Kaur & Deol, 2020).The class I bacteriocins are characterized by post-translational modifications that result in nonstandard amino acids such as lanthionine, labyrinthine, and dehydrobutyrine (Pircalabioru et al., 2021)(Kaur & Deol, 2020).They undergo extensive post-translational modifications that are required for antimicrobial activity and auto-inducers activity, which are responsible for the upregulation of their own expression (Islam et al., 2012). Table 1.1 shows three different bacteriocin classes of Gram-positive bacteria (Zimina et al., 2020).

Table 1.1: Different bacteriocin classes of Gram-positive bacteria (Zimina et al., 2020).

Class	**Group**	**Distinctive Characteristic**	**Activity**
I	Lantibiotics	Residues (methyl)lanthionine	Antibacterial or inhibitory activity against
	Lipolantins	N-terminal fatty acid and avionin fragment	multidrug-resistant Staphylococcus aureus, Streptococcus spp., Clostridium difficile, Bacillus sp. and Enterococcus sp.
	Thiopeptides	6-membered nitrogen heterocycle, azole rings	
	Botromycins	Macrocyclic amidine, decarboxylated C-terminal thiazole, β-methylated residues	
	Linear azole-containing peptides	thiazole and (methyl)oxazole rings, linear back bone	
	Sactibiotics (sactipeptides)	Disulfide α-carbon bridges	
	Lasso peptides	Cyclization of an N-terminal amine into a γ-acid	
	Cyclic bacteriocins with a “head-to-tail” connection	Cycling from N-terminus to C-terminus	
	Glycocins	Glycosylated residues	Antibacterial or inhibitory activity against Escherichia coli, Listeria monocytogenes, Staphylococcus epidermidis, Klebsiella pneumonia, and Staphylococcus aureus
II	YGNG-motif containing bacteriocins	Consensus YGNG-motif, at least one disulfide bridge	
	Linear two-peptide bacteriocins	Synergy of two peptides	
	Leaderless bacteriocins	Lack of a leader peptide	
	Other linear bacteriocins	Non-YGNG-like linear peptides	
III	Bacteriolysins	Large lytic polypeptides,	
	Non-lytic bacteriocins	Large non-lytic polypeptides	Broad-spectrum inhibitory activity
	Tailocins	Multiprotein complex, a structure similar to a phage tail	activity against Staphylococcus aureus, Streptococcus agalactiae, Pseudomonas aeruginosa, and Gardnerella vaginalis

Class I bacteriocins have inhibitory activity against multidrug-resistant *Staphylococcus aureus, Streptococcus* spp., *Clostridium difficile, Bacillus* sp. and *Enterococcus* sp. Class 1 bacteriocins are referred to as lantibiotics and they include examples such as Nisin and Lactisin (Zacharof and Lovitt 2012). Nisin is a popular lantibiotic that is used in the food industry as a preservative (Lobo-Ruiz and Tulla-Puche 2018). Maricic et al. (2016) described the functional lantibiotic gene locus found in pneumococci. Maricic et al. (2016) noted that *S. pneumoniae* and *S. mitis* have *pld* genes that are responsible for the production of pneumolancidin. According to Maricic et al. (2016), the *pld* locus encodes a set of four unique inhibitory peptides, where only one is not needed for antibacterial action. Maricic et al. (2016) noted that the strains that produce pneumolancidin inhibited all the pneumococci strains tested.

Lantibiotics are highly promising for use as potential therapeutics for many reasons. First, they are quite potent; they can inhibit bacterial growth at low concentrations. Second, lantibiotics demonstrate two types of mechanism of action, which make it more difficult to develop resistance for the target bacteria. These key features could prove useful in the treatment of antibiotic-resistant bacteria. However, lantibiotics are notoriously difficult to synthesize, and current methods of purification from the host cells are laborious (van Dijk, et al., 2018). The understanding of how the production of lantibiotic peptides is regulated within the cell could be useful to improve output and purification. Also, the characterization of the protein structure and how this structure relate to the overall function will be fundamental to our understanding of these entities as medicines and will provide a new insight into the design of novel antimicrobials.

The second class of bacteriocins (Class II) have a molecular weight of not less than five but not more than 10 kDa. The Class II bacteriocins are non-modified small peptides that are resistant to pH changes and stable across varying temperatures (Belguesmia et al., 2011;

Pircalabioru et al., 2021). They are made up of five subtypes that include two peptides bacteriocins (Class IIb), pediocin- like (Class IIa), circular (Class IIc), and nonpediocin-like linear (Class IId). Class II bacteriocins ST22Ch, ST153Ch, and ST154Ch, produced by *Latilactobacillus sakei* strains (ST22Ch, ST153Ch, and ST154Ch) have inhibitory activity against *Escherichia coli, Listeria monocytogenes, Staphylococcus epidermidis, Klebsiella pneumonia*, and multidrug-resistant *Staphylococcus aureus* (Darbandi et al., 2022a).

The third group of bacteriocins are Class III, contrary to class I and class II bacteriocins, class III bacteriocins are large peptides (>30 kDa), and may be heat-labile lytic or non-lytic (Simons et al., 2020). Bacteriocins such as zoocin A, lysotaphin or helveticin J and V are included in this group (Simons et al., 2020). These bacteriocins have an antibacterial activity linked to enzymatic activity (e.g., endopeptidase), leading to the disruption of the bacterial cell wall.

Class III bacteriocins have a broad-spectrum inhibitory activity against *Staphylococcus aureus, Streptococcus agalactiae, Pseudomonas aeruginosa,* and *Gardnerella vaginalis* (Garcia-Gutierrez et al., 2019; Ibrahim, 2019). The fourth group of bacteriocins (Class IV) are complex proteins that some researchers classify as hydrolytic polypeptides (Suganthi & Mohanasrinivasan, 2015; Pircalabioru et al., 2021).

1.2.4.1.2 General functions of bacteriocins.

Bacteriocins can be used for various functions. bacteriocins can also be used as antiviral agents with the example being Enterocin CRL35 used against *Herpes simplex virus* (Wachsman et al. 2003). bacteriocins can also act as microbiota regulators eliminating intestinal colonisation by antibiotic-resistant bacteria (Kommineni et al. 2015; Drider et al. 2016). bacteriocins can also be used as plant growth promoters (Subramanian and Smith 2015). Some bacteriocins have shown selective cytotoxic activity toward cancer cells while

sparing normal cells, and therefore, potentially useful as anticancer agents (Kaur & Kaur, 2015)(Chaudhary and Munshi 1995; Zhu et al. 2008).

Bacteriocins are also used as antimicrobial agents or complementary agents to improve antimicrobial performance because they are not inhibited by antimicrobial agents (Darbandi et al., 2022b). Evidence shows the use of bacteriocins along with antibiotics to fight infections caused by common nosocomial pathogens such as *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter Baumanii,* and *Pseudomonas aeruginosa* (Naghmouchi et al. 2012; Al Atya et al. 2016). Evidence from *in vitro* assays report antibacterial activity of circular bacteriocin AS-48 against multidrug-resistant *Mycobacterium tuberculosis* (Aguilar-Pérez et al. 2018), pentocin JL-1 isolated from Lactobacillus pentosus against MDR *S. aureus* (Jiang et al. 2017), and carbapenem-resistant *Klebsiella* species (Denkovskienė et al. 2019). However, the interaction between bacteria and the bacteriocins can lead to adaptive responses from the target bacteria (Kumariya et al., 2019). For example, *S. pneumoniae* has developed resistance towards the bacteriocin nisin through D-alanylation of teichoic acid (McBride & Sonenshein, 2011).

According to Gradisteanu Pircalabioru et al. (2021), bacteriocins have compelling features that make them appealing antibiotic candidates. One of the features is the fact that a single molecule of bacteriocins can target and eliminate an invading target cell. Secondly, bacteriocins have a wide range biological activity; facilitating action against a wide range of causing microorganisms causing human and animal diseases. Thirdly, bacteriocins act quickly in inhibition or inhibiting the target organisms. It should also be noted that bacteriocins do not face the challenge of inactivity due to mutations that occur in the target organisms as the case with conventional broad-spectrum antibacterial agents. Finally, bacteriocins are stable across a wide range of ecological conditions due to the diversity in

structure and high-level post-translational modification making them more stable (Gradisteanu Pircalabioru et al., 2021).

Compared to antibiotics, bacteriocins have various advantageous properties (Hols et al., 2019). The stability of antibiotics to temperature changes and pH variation is low but bacteriocins especially Class I and Class II have high stability (Perez et al., 2014; Hols et al., 2019). Some antibiotics have high cytotoxicity while bacteriocins have low cytotoxicity (Dicks et al., 2018; Torres et al., 2018). Unlike antibiotics, bacteriocins are relatively easier to produce because they can easily be chemically synthesised especially for Class II bacteriocins (Torres et al., 2018; Hols et al., 2019). Because of their peptide backbone, bacteriocins can be sensitized to proteases. Thus, they would display a lower biological half-life in nature and organic environments. This is considered as an asset to dampen the emergence of resistance, since low remnant and sublethal concentrations of toxins are mutagenic and favor the progressive acclimation of an initial clonal population (Hols et al., 2019). Therefore, can effectively treat multi-resistant infections. unlike antibiotics (Hols et al., 2019). However, bacteriocins are relatively of high-molecular weight and have poor environmental resilience compared to antibiotics (Hols et al., 2019). In addition, pathogenic microorganisms can potentially develop immunity against bacteriocins and therefore, bacteriocins may gradually lose their antimicrobial potency (Pérez-Ramos et al., 2021).

Previous studies have demonstrated that *S. pneumoniae* mediates intra-species competition by the release of bacteriocins and immunity proteins encoded by the highly variable *blp* cassette (Son *et al.*, 2011). Recently, bacteriocin-producing microorganisms have attracted significant attention because of their ability to inhibit the growth of other bacteria, and the producer strain has a dedicated immunity system that protects it against its bacteriocin. Comprehension of the mechanism behind inhibition and protection from these studies may allow the future generation of bacteriocins as pharmaceutical drugs for the treatment of

infections. The development of bacteriocin into antimicrobial agents is crucial due to the dramatic increase in antibiotic-resistant bacteria (Simons et al., 2020).

The approach through which bacteriocin influence the association between producers and the neighbouring cells is complex (Lehtinen et al., 2020). Various work on the dynamics of bacteriocin systems indicates that the two-strain model that include bacteriocin-producing strain and strains susceptible to the bacteriocin toxin in hand, does not provide adequate understanding of the dynamics (Lehtinen et al., 2020). According to Levin (1988) and Durrett and Levin (1997) the producer strain or the non-producer strain can be out-competed depending on the effectiveness of the bacteriocin and the cost of producing it. Lehtinen et al. (2020) observed that the bacteriocin producer can still be outcompeted by the non-producers if bacteriocin production is inefficient or if the target non-producers have developed significant immunity against the produced bacteriocins. Durrett and Levin (1997) noted that co-existence could be attained if there is a third strain (immune strain) that is not bacteriocin producer (or produces the bacteriocin in negligible and ineffective quantities) and is also not susceptible to the bacteriocins produced by the producers. If the cost of bacteriocin and immunity production is not too higher the producers out-compete the non-producers (Lehtinen et al., 2020). In a situation where the non-producers are eliminated by the toxins produced by bacteriocin producing strains, the resources freed up will be used by the producers and the immune strains. According to Lehtinen et al. (2020), the total cost of the interaction is higher to the producers compared to the immune strain that only need to meet the cost of immunity. In this interaction, the immune strains benefit from the freed resources but does not incur the cost of producing toxins, which lead to their proliferation (Durrett & Levin, 1997; Kerr et al., 2002). It should however be noted that for *S. pneumoniae* the duration of colonisation and aspects related to transmission can affect the overall bacterial population

(Turner et al., 2011; Kamng'ona et al., 2015; Hjálmarsdóttir et al., 2016; Lehtinen et al., 2020).

1.2.4.1.3 Mechanism of action of bacteriocins against competing organisms

1.2.4.1.3.1 Bactericidal action through pore formation

One of the well documented mechanisms of action of bacteriocins as antimicrobials is the formation of pore on the cell wall of the target cells or through membrane perturbations (Etayash et al., 2016; Preciado et al., 2016). bacteriocins act on the bacterial cell wall with the help of a docking molecule such as lipid II (Héchard & Sahl, 2002; Kumariya et al., 2019). The class I bacteriocins such as nisin have been shown to act on the target cell by forming pores assisted by a docking molecule and through interaction with cell wall precursor lipid II (Sharma et al., 2021). The interaction between nisin and the lipid results in the formation of a pyrophosphate cage on the head group and the flexible hinge region of the lipid causes the translocation of C-terminus resulting in the cell wall perforation (Prince et al., 2016). The loss of intracellular material eventually leads to death (Kumariya et al., 2019). In *S. pneumoniae*, pneumolysin also inhibit the target cells through pore formation (Rai et al., 2016; Vögele et al., 2019).

However, other bacteriocins do not need docking molecules but instead use transporters such as ABC transporter. However, it should be noted that the docking molecules play an important role in stabilising the pores made by the bacteriocin on the membranes (Moll et al., 1999). Nisin producers (*Lactococcus lactis*) have immunity protein that protects its cell wall against the action of nisin by protecting lipid II (Alkhatib et al., 2014).

1.2.4.1.3.2 Inhibition of cell wall biosynthesis

Bacteriocins can inhibit the biosynthesis of the cell wall by disrupting transmembrane peptidoglycan transport to the cell wall site (Sharma et al., 2021) (Hernández-González et al., 2021). Peptidoglycan monomers are required for polymeric biosynthesis of bacterial cell wall

component. However, peptidoglycan monomers are initially synthesized within the cytoplasm and must be transported to the cell wall through its a hydrophobic carrier, lipid II. Bacteriocins bind competitively to lipid II, reducing molecules of peptidoglycan monomers available for binding to the lipid II hydrophobic carrier for onward transmembrane transport. This disrupts cell wall formation (Bauer & Dicks, 2005; Hernández-González et al., 2021).

1.2.4.1.3.3 Inhibition through binding of DNA

Bacteriocins can cause cell death by binding DNA resulting in disrupted protein synthesis (Sharma et al., 2021). Miao et al. (2016) reported that bacteriocins could cause rapid cell death by damaging and disrupting the genomic DNA through binding. Miao et al. (2016) based their observations on the assessment of action of peptide F1 against *S. aureus*. Duan et al. (2014) noted that bacteriocin haloemodin has inhibitory activity against vancomycin-resistant *Enterococcus faecium* and methicillin-resistant *S. aureus*. Duan et al. (2014) noted that bacteriocin haloemodin act by disrupting the DNA through the inhibition of DNA gyrase, the enzyme that catalyses super-coiling of the DNA. Yi et al. (2020) observed that bacteriocin BM1157 inhibited the growth of *S. aureus* by disrupting the DNA replication and repair. Maldonado-Barragan et al. (2013) noted that class IId bacteriocin, garvicin A has bactericidal effects. The researchers noted that the bacteriocin inhibits septum formation in *Lactococcus garvieae* cells. Maldonado-Barragan et al. (2013) noted that garvicin A showed high specificity towards *L. garvieae* and not other beneficial bacteria, indicating its potential importance as an antimicrobial.

1.2.4.1.3.4 Inhibition through disruption of ATP synthesis

Bacteriocins can also inhibit the growth of the target cell by disrupting ATP synthesis or causing its release from cells (Sharma et al., 2021). The bacteriocins targeting the ATP synthesis act on the ATP synthase and adenine nucleotide translocase (Sharma et al., 2021). Christensen and Hutkins (1992) noted that bacteriocins also increased permeability of cell

membranes through formation of ion selective pores which disrupt proton motive force and deplete intracellular ATP.

1.2.4.2 Fratricide

Fratricide is used by competent pneumococcus not only to eliminate direct competitors but also to acquire nutrients and DNA released by non-competent lysed cells (Håvarstein *et al.*, 2006). Initially, it was hypothesised that CSP might influence co-colonisation because of fratricide-mediated exclusion (Claverys *et al.*, 2007). This hypothesis was valid because *S. pneumoniae* populations are mainly divided into two phenotypes (pherotypes) that express and respond to distinctive types of CSP. Two studies evaluated the impact of CSP types on the co-existence of *S. pneumoniae* in humans, and could not prove this hypothesis (Valente *et al.*, 2012; Vestrheim *et al.*, 2011). In a study by Vestrheim et al. (2011), the distribution of pherotypes in sets of co-colonising pneumococcal strains was not different from the expected proportions based on the overall pherotype distribution in the strain collection. This demonstrated that pneumococci belonging to distinct pherotypes can co-exist in a stable and independent way in the nasopharynx. These findings suggest that fratricide committed by competent cells does not affect the within-host competition between strains of different pherotypes (Valente et al., 2012).

1.2.5 Bacteriocin production in S. pneumoniae

To compete with surrounding bacteria found in the nasopharynx, the pneumococcus secretes bacteriocins. *In vivo* studies of animal models of *S. pneumoniae* colonisation have demonstrated that bacteriocin production provides a competitive advantage during their establishment on the mucosal surfaces (Dawid *et al.*, 2007; Son et al., 2011). These studies were performed by simultaneously inoculating mixtures of competing strains at 1:1 ratio in the nasopharyngeal tract of a murine mouse. The colonisation dynamics are more likely to be far more complex in the humans' nasopharyngeal tract. Although co-colonisation by distinct

S. pneumoniae strains is quite common, the relative proximity of these strains within the human host remains unknown. Based on extensive genomic studies, such strains can support genetic exchange (Chewapreecha *et al.*, 2014; Donkor *et al.*, 2011). The pneumococcus inhibits sensitive strains and has a fitness advantage in both *in vitro* assays and competitive colonisation in *in vivo* assays of a mouse nasopharynx (Dawid et al., 2007; Wholey *et al.*, 2016).

1.2.6 The blp locus in *S. pneumoniae*

The major family of bacteriocins produced by pneumococcus is called pneumocin. The pneumocin locus is called *blp* and is found in all sequenced strains of pneumococcus (Bogaardt C et al. 2015). The *blp* locus in *S. pneumoniae* has been associated with bacteriocin production and has been shown to play a role in intra-species competition in a murine model of colonisation (Dawid et al., 2007). However, it should be noted that although pneumococci produce various bacteriocins, only a few shows activity against competing bacteria. Examples of inhibitory bacteriocins produced by pneumococcal include blppneumocins, CibAB bacteriocin pair, and pneumolancidins (Bogaardt et al., 2015; Maricic et al., 2016). The *blp* locus is involved in encoding the factors required for regulation, production, and secretion of bacteriocins and their corresponding immunity proteins.The *blp* locus also has genes that encode the two-component regulatory system (BlpRH) that play a role in the production of BlpK immunity proteins that offer protection against the bacteriocins (Wholey et al., 2019). A two-component regulatory system, consisting of a histidine kinase (BlpH) and a response regulator (BlpR), controls the *blp* locus. The *blp* system is activated when the signalling peptide, BlpC, reaches a certain level and binds to its cognate BlpH receptor (Dawid et al., 2007; Reichmann and Hakenbeck, 2000). The BlpH then binds to the BlpR and activates it. The *blp locus*embraces genes that encode a typical three-component regulatory system (Blp-CRH), an ABC transporter (BlpAB), and conserved proteins (Son et al., 2011), which may

contribute to bacteriocin immunity (BlpYZ and SPO547). The predicted pneumococcal bacteriocin peptides, also known as pneumocins, show homology to class-II bacteriocin precursor peptides (Bogaardt et al., 2015). The peptide pheromone, BlpC, consists of a conserved N-terminal leader sequence linked to a double-glycine motif and alanine/glycine-rich mature peptides (Bogaardt et al., 2015). These molecular characteristics are typical of bacteriocins of the Gram-positive *S. pneumoniae* (Bogaardt et al., 2015) (Cook & Federle, 2014; de Saizieu et al., 2000). The pneumococcal peptide is cleaved and transported out of the bacterial cell via BlpAB. When levels of BlpC are sufficiently high, BlpC triggers activation of the histidine kinase BlpH. This results in the phosphorylation and activation of the regulator component, BlpR, a response regulator (Son et al., 2011). The activation of the regulator BlpR results in upregulation of the entire blp locus including the bacteriocin/immunity region (BIR) which encodes the bacteriocins and their cognate co-transcribed immunity proteins (Bogaardt et al., 2015; de Saizieu *et al.*, 2000; Reichmann and Hakenbeck, 2000).

Upstream of the blp locus, the locus embraces open reading frames for a typical two-component regulatory system (BlpR and BlpH), a small peptide pheromone (BlpC), and a dedicated ABC transporter system (BlpA nd BlpB). The ABC transporter component is thought to recognise the N terminus of both the pheromone and the bacteriocins and to transport these peptides across the cytoplasmic membrane, concurrently with cleavage at a conserved double-glycine motif. The cleaved extracellular BlpC can then attach to the sensor kinase, BlpH, which allows the activation of BlpR and the upregulation of the entire gene via binding to a consensus sequence within each promoter (Dawid et al., 2007). Transcriptional studies of the *blp* locus in the two fully sequenced pneumococcal strains R6 and TIGR4 demonstrated that application of chemically synthesised BlpC resulted in upregulation of several operons only within the *blp* locus, including those encoding the regulatory proteins,

transport apparatus, and putative bacteriocins (de Saizieu et al., 2000). For downstream genes within the locus, the levels of transcripts encoding BlpXY and BlpZ were also upregulated by the addition of the pheromone BlpC. This operon encodes proteins thought to be involved in immunity. Studies on several pneumococcal strains demonstrated that there are at least four different pheromones secreted and that each pheromone is specific for its cognate BlpR and BlpH protein (de Saizieu et al., 2000; Reichmann and Hakenbeck, 2000).

The *blp* genes responsible for the production and regulation of *blp* bacteriocins are organised in gene clusters and have several operons, typically flanked by the genes ecsB and a putative choline kinase located at approximately 400-600 kb from the origin of replication. Bacteriocin bacteriocin expression is regulated by a classical quorum sensing two-component regulatory system (de Saizieu et al., 2000), that is conserved among diverse *S. pneumoniae* genomes (Pinchas *et al.*, 2015). BlpC is the inducer peptide, that embraces an N-terminal double-glycine motif and is putatively processed and exported by an ABC transporter system, BlpAB. A study showed that an engineered laboratory strain with intact BlpAB efficiently processes BlpC but not in the absence of BlpA (Kochan and Dawid, 2013). BlpC binds specifically to a membrane-located histidine protein kinase (BlpH) when it reaches a certain threshold (Pinchas et al., 2015). This activates the DNA binding response regulator BlpR by phosphorylation (BlpR-P). The phosphorylated BlpR-P binds to its specific cognate sequence sites in the promoter regions of the *blp* locus to activate their expression. The *blp* gene cluster also embraces two genes of unknown function (blpS and blpT) along with genes encoding (putative) bacteriocins (known as *blpD, blpE, blpI, blpJ, blpK, blpM, blpN, blpO, blpQ*, pncT and *pncW*) and their cognate immunity genes (known as *blpL, blpX, blpY, blpZ* and *pncP*) (Bogaardt et al., 2015). Like blpC, bacteriocins contain an N-terminal double-glycine leader sequence that is cleaved and processed upon export via the ABC-transport system. The *blp* region is a highly variable region and the number of bacteriocin genes differs significantly

between different pneumococcal strains (Lux *et al.*, 2007; Son et al., 2011). However, a superficial analysis indicates that the *blp* cluster is intact despite significant variation in this region even in strains with degenerated BlpAB.

Evidence indicates that there is variability in the content of the *blp* locus across the different pneumococcal genomes (Wholey et al., 2019). The variability is evident in the major alleles of the gene encoding peptide pheromone where four variants exist ($BlpC_{164}$, $BlpC_{R6}$, $BlpC_{6A,}$ and $BlpCT_4$) (Miller et al. 2016). It should be noted that the two-component regulatory system can maximally respond to specific variant of peptide pheromone (Pinchas et al., 2015). According to Wholey et al. (2019) the variability in the *blp* locus is also characterized by varied bacteriocin genes and the associated immunity genes, which explains the variation in the competition approaches among pneumococcal isolates.

Wholey et al. (2019) characterized the competitive pneumocin peptides of *S. pneumoniae* and predicted the role of the various peptides in promoting bacterial competition. Wholey et al. (2019) noted that the pneumocins are made up of BlpK, BlpI and BlpJ peptides. They noted that BlpIJ function as a two-peptide pneumocin while BlpK functions as single-peptide pneumocin. Wholey et al. (2019) noted that BlpIJ combination has inhibitory action against a range of Gram-positive microbes (including; *S. pyogenes* strains, *S. mitis, S. oralis,* and *Enterococcus faecalis*). However, the inhibitory activity of BlpIJ combination is dependent on Blp transporter and only few strains meet the conditions. On the contrary, BlpK pneumocin is found in most of the strains.

The mechanism for Blp peptide export is likely the paralogous quorum-based two-component signalling system regulating competence for natural transformation (comCDE) This has already been demonstrated using *S. mutans* (Tian et al., 2009). The export of a quorum-sensing peptide (QSP) mediates quorum component regulation via an ABC transporter ComAB, followed by QSP concentration-dependent activation of the downstream late

competence genes. The *blp* genes have been shown to be weakly upregulated during induction of pneumococcal competence (Dagkessamanskaia *et al.*, 2004). Competent *S. pneumoniae* also express CbpD and CibAB, a murein hydrolase and a two-peptide bacteriocin that both causes lysis of non-competent cells (Guiral *et al.*, 2005). A diagram summarising the quorum-sensing system using *S. mutans* is shown Figure 1.2 below.

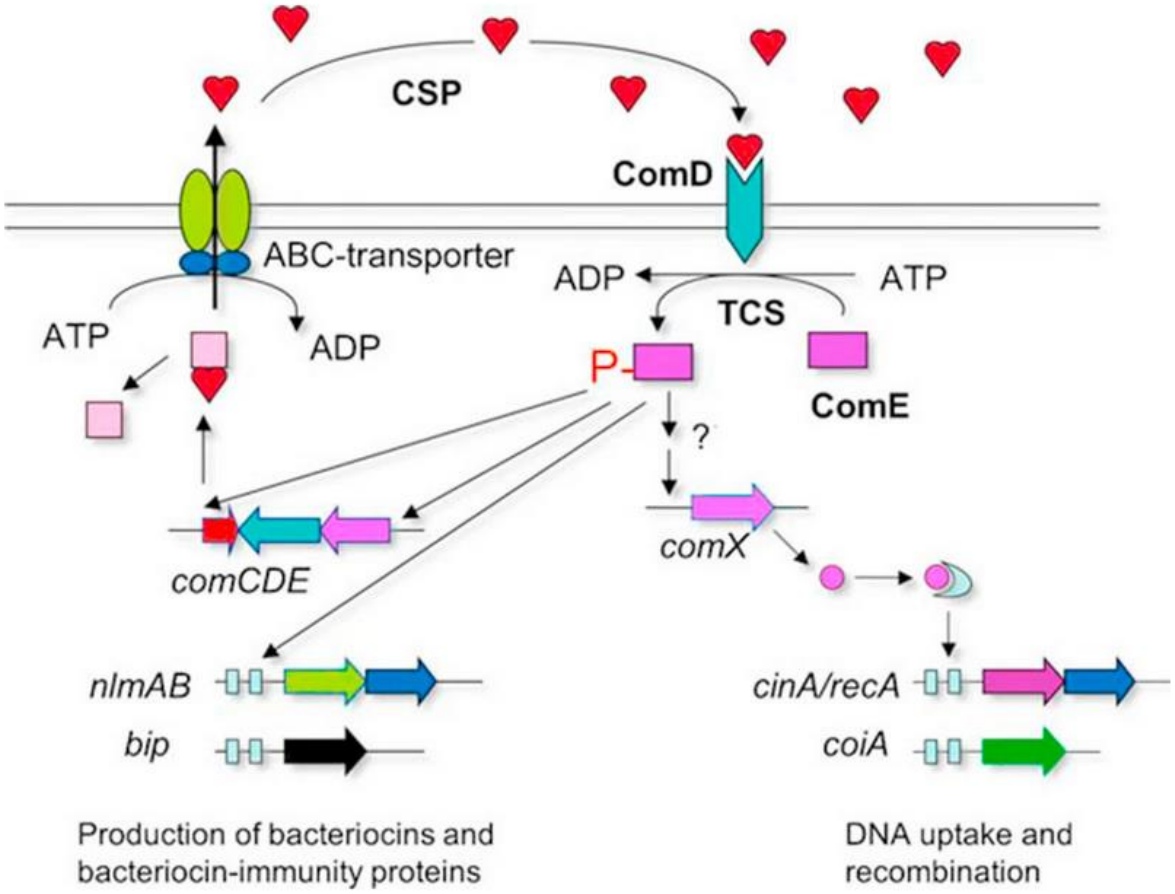

Figure 1-2: A schematic illustration of the quorum-sensing system and its controlled cooperative activities in Streptococcus mutans. The CSP is the signalling peptide, which induces quorum-sensing cascade activated when a certain critical concentration is reached. This subsequently triggers the production of copious amounts of bacteriocins and genetic competences, which culminates in the inhibition of other rival species, DNA release, and gene exchange (Tian et al., 2009).

1.2.7 Competence regulatory system in *S. pneumoniae*

The competence regulatory system is susceptible to environmental factors including pH and exposure to certain antibiotics (Charpentier *et al.*, 2012; Slager *et al.*, 2014). Previously, it was known that only cell density activates the competence induction. Recently, it was found

that not only cell density but also any kind of stress like antibiotics and pH can also induce competence (Claverys et al., 2007).

S. pneumoniae is a naturally competent bacteria as it can exploit many of the genetic materials available to it within the nasopharynx (Bogaert et al., 2011; Könönen *et al.*, 2002) (Aniansson G et al. 1992) (Könönen E et al. 2002) (Bogaert D et al. 2011). Natural competence makes pneumococcus capable of taking up new genetic material through horizontal gene transfer and recombination. Several studies have shown that recombination happens with great frequency in pneumococcus lineages that are globally distributed (Croucher *et al.*, 2011), geographically isolated (Chewapreecha et al., 2014), and even confined to a single patient (Hiller *et al.*, 2010).

1.2.8 Competence induction

When CSP reaches a critical concentration in the extracellular medium, in the liquid culture of *S. pneumoniae*, it stimulates competence (Shanker and Federle, 2019). This might be due to cell density-dependent quorum sensing mechanism (Moreno-Gámez *et al.*, 2017). The CSP binds to a specific receptor which is a membrane histidine kinase, ComD, and turns it on; The activated ComD trans-phosphorylates ComE. The phosphorylated ComE activates transcription of ComC, and results in more production and accumulation of CSP levels. Thus, it stimulates the competence process. In addition, the activated ComE activates the production of an alternative sigma factor which is the product of ComX. ComX, on the other hand, activates the transcription of late competent genes that code proteins that are responsible for the uptake, binding, and recombination of extracellular DNA. Competence provides DNA uptake to serve as material for genetic exchange, DNA repair, and acquire toxins and nutrients. ComX activity is necessary for the expression of many genes required for competence (Moreno-Gámez et al., 2017; Shanker and Federle, 2017). This conserved Ceb element is also near the ComAB operon as well as the ComCDE operon. ComE-P binds

directly at the Ceb in the promoter region (PCeb) of these operons to activate gene expression. This results in the amplification of the core components of the CSP signal transduction pathway is illustrated in Figure 1.3 (Shanker and Federle, 2017). Previously, it was known that only cell density activates the competence induction. Recently, it was found that not only cell density but also any kind of stress like antibiotic and pH can also induce competence. Therefore, this indicates that CSP acts as a general stress response pheromone, and competence is a general stress response mechanism (Claverys et al., 2006).

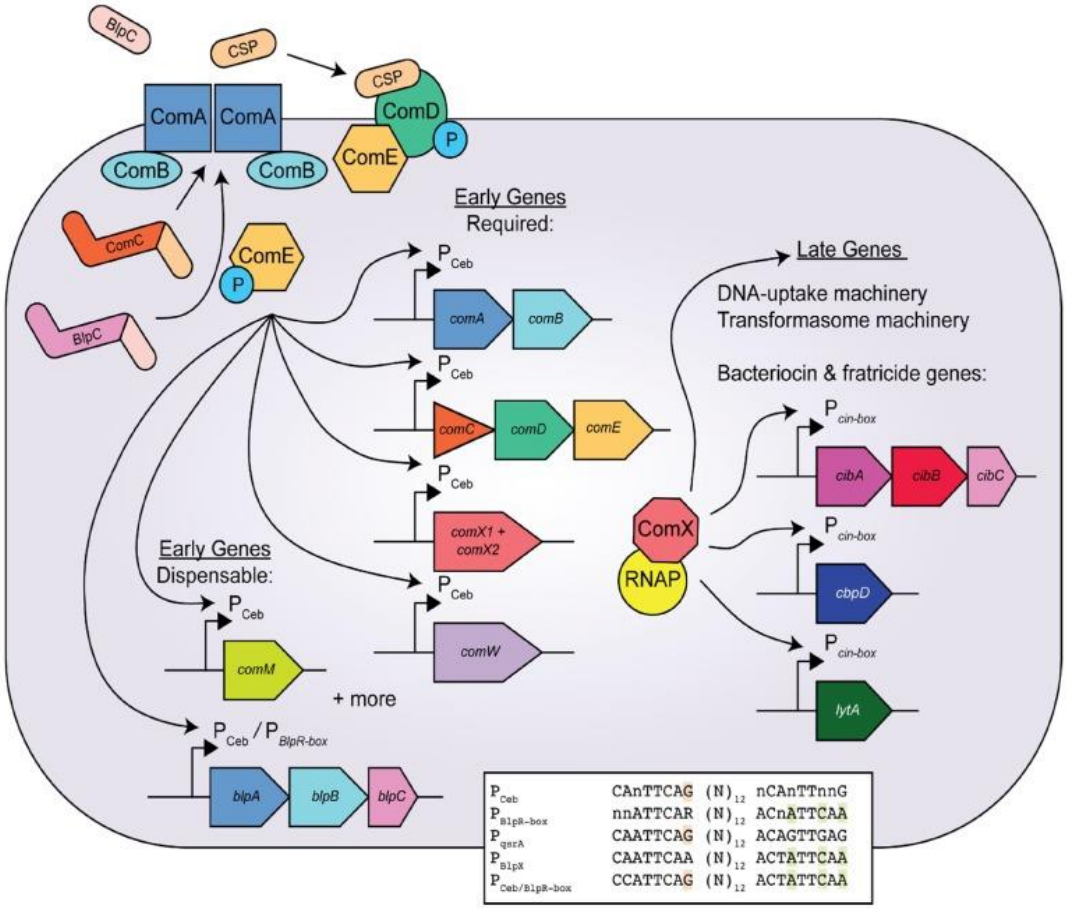

Figure 1-3: The ComABCDE quorum sensing (QS) pathway of *S. pneumoniae* (Shanker and Federle, 2019).

The proteolytic enzyme called HtrA, trypsin-like enzyme belonging to peptidase S1C family, play an important role in the regulation of bacteriocin production in *S. pneumoniae* (Marquart, 2021). HtrA is located on the cell surface of *S. pneumoniae* (De Stoppelaar et al., 2013). At high temperatures of about 42 degrees centigrade the HtrA molecules functions as a protease but at normal temperatures it functions as a molecular chaperone (Spiess et al., 1999). The

study carried out (Ibrahim et al., 2004) suggested that HtrA has an important role in the pneumococcal competence. Ibrahim et al., (2004) observed that the cells that were deficient in HtrA had reduced transformation efficiency. However, other researchers have shown that the protease breakdown competent stimulating peptides (Ibrahim et al., 2004). Evidence suggests that the variants of *S. pneumoniae* with deleted *htrA* show increased production of pneumocins. According to Kochan and Dawid (2013), HtrA participates in the regulation of pneumocin production by controlling the secretion of bacteriocin peptide pheromone (BlpC). Reduced production of bacteriocin peptide pheromone limit the sensing by neighbouring cells and therefore reducing the production of pneumocin (Kochan & Dawid, 2013).

1.2.9 Interplay between competence regulatory system and bacteriocin production

To date, two major competence-mediated bacteriocins implicated in *S. pneumoniae* intra-species competition, include lantibiotics, and fratricide (Dawid et al., 2007; Guiral et al., 2005; Håvarstein et al., 2006). The two bacteriocin systems were historically thought of as independent systems. However, recent studies have demonstrated that these systems are regulated co-ordinately. This suggests that under some conditions, fratricide and bacteriocin production may work in synergy to target the surrounding cells (Kjos et al., 2016; Wholey et al., 2016). Figure 1.4 shows competence and Blp regulation in *S. pneumoniae*. The blp locus regulates bacteriocin production in tandem with the competence system (de Saizieu et al., 2000). Briefly, a small peptide pheromone, called BlpC, is processed and secreted by the BlpAB transporter complex. The mature form of BlpC, now, the CSP, accumulates extracellularly and when a sufficient threshold concentration is reached, it signals through the ComDE to induce competence (Wang et al., 2018).

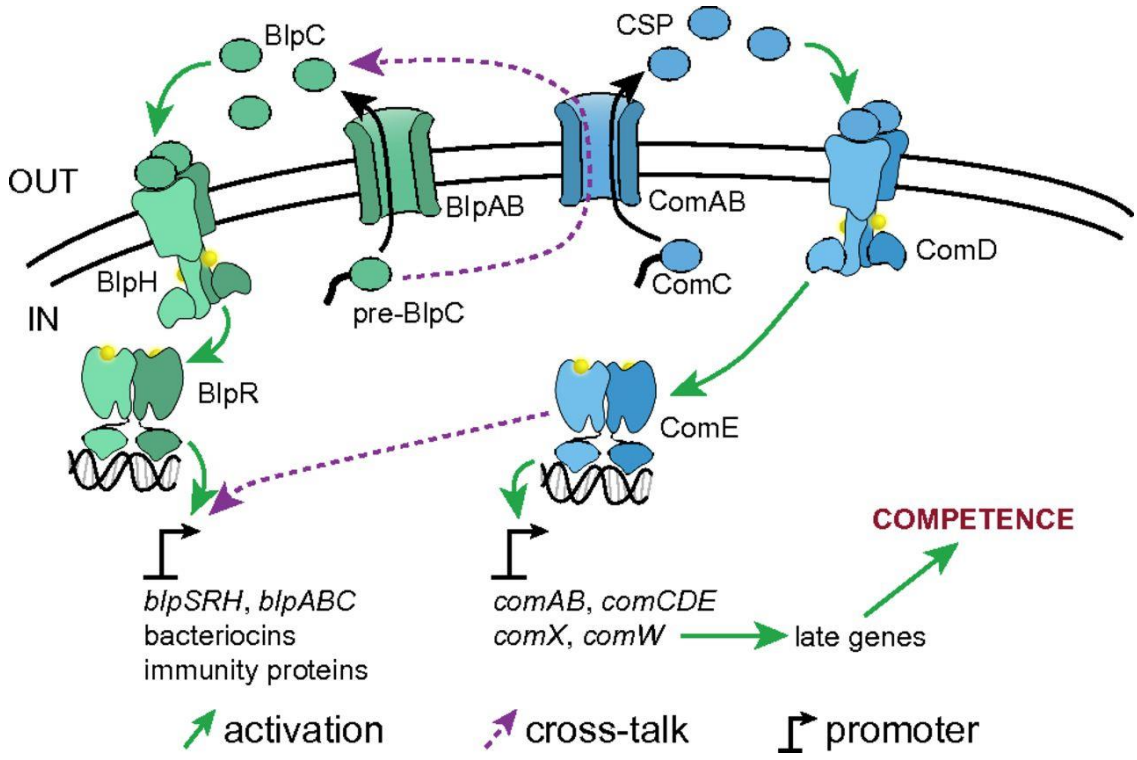

Figure 1-4: The competence and Blp regulation in S. pneumoniae (Wang et al., 2018).

At the centre of the Com and blp systems lie the peptide transporters ComAB and BlpAB, both belong to the superfamily of ABC transporters. ComAB and BlpAB are composed of A and B subunits. The role of the B subunits, encoded by ComB and BlpB, remains unclear. However, the A subunits, encoded by ComA and BlpA, embrace all the known functional domains. Each A subunit harbours an N-terminal part followed by the channel-forming transmembrane domain and a C-terminal nucleotide-binding domain (NBDs) (Håvarstein *et al.*, 1995). The peptidase domain catalyses the beginning of transport called ‘’processing’’: the cleavage of the peptide substrate immediately C-terminal to a conserved double-glycine (GG) motif, sometimes also GA or GS, thereby removing the substrate’s N-terminal signal sequence (Furgerson Ihnken et al., 2008). Mutations in the GG motif results in either abolished or severely inhibited substrate cleavage (Furgerson Ihnken et al., 2008). This explains, at least in part, why there is a near-perfect conservation in this family of peptides.

Post-processing, the mature peptide is secreted out of the cell by using energy provided by the ATP-hydrolyzing NBDs. While ComAB can create both ComC and BlpC and this has revealed some degree of promiscuity in its substrate selection process, it is yet unknown if BlpAB shares this property or just how far it extends (Håvarstein et al., 1995; Kotake *et al.*, 2008).

Study of the locus in the two fully sequenced pneumococcal strains R6 and TIGR4 demonstrated that the application of chemically synthesized BlpC leads to upregulation of a number of operons only inside the locus, including those encoding the regulatory proteins, transport apparatus, putative bacteriocins (de Saizieu et al., 2000). The transcript analysis of a downstream operon encoding BlpXY and BlpZ was also upregulated by the addition of BlpC. This operon encodes proteins thought to be involved in the production of immunity proteins. A study of several pneumococcal strains showed that there are at least four different pheromones produced and that each is specific for its cognate BlpR/BlpH protein (de Saizieu et al., 2000; Reichmann and Hakenbeck, 2000). To protect itself from a bacteriocin associated suicide as well as to defend against a wide variety of bacteriocins of other competing strains *S. pneumoniae* has repertoire of putative immunity genes, including BlpF, BlpG, BlpL, BlpP, BlpX, BlpY, BlpZ, pncG, pncM, and pncP. The possession of multiple immunity genes could simply mean the pneumococcus has an increased repertoire of bacteriocin arsenal and broadened immunity. These self-protection genes are also activated by the response regulator, BlpR (Bogaardt et al., 2015).

Wholey et al. (2019) observed that the isolates of pneumococci that showed immunity had BlpU4/5 immunity proteins, and intact BlpAB+ system. Miller et al. (2016) and Son et al. (2011) noted that only 25 % of the pneumococci strains encoded a full length, functional BlpA transporter (BlpA+), while the remaining majority BlpA non-functional (BlpANF) strains exhibit conserved frame shift (BlpAFS) mutation or significantly large deletion in the

blpA transporter gene. Wholey et al. (2019) also observed that only 4% of the strains (from the Maela and Boston collection) were BlpA+ intact and could produce pneumocin BlpIJ, suggesting a broad spectrum of inhibition by the pneumocin. Miller et al. (2016) further argued that the deletion rendered the strains unable to secrete the peptide pheromone and the bacteriocin. However as demonstrated by Wholey et al. (2019), the $blpK_{out}$ /$pncF_{out}$ operon play a key role in the production of immunity proteins and does so even in the absence of the regulatory *blp* genes. The researchers reported that strains with deleted *blp* genes were still able to show BlpK specific immunity, which was due to $PncF_{out}$operon.

Evidence indicates that *comAB* genes can also produce pneumocins such as in$BlpA_{NF}$ bacteria (Kjos et al., 2016; Wholey et al., 2016). Wholey et al. (2019) also showed that *comAB* genes can produce pneumocins but are limited by short period of competence. However, it should be noted that compared to $BlpA_{NF}$ bacteria, the BlpA+ are better equipped to compete (Wang et al., 2018). $BlpA_{NF}$ bacteria that rely on *comAB* genes have also been reported to be susceptible to inhibition caused by bacteriocins (Wang et al., 2018).

1.3 Thesis Aims and Objectives

The central goal of the thesis is to characterize the bacteriocin (Blp) locus in *S. pneumoniae* TIGR4 strain and understand the mechanism by which it works. This study was prompted by previous findings where TIGR4 did not produce bacteriocins despite having a complete locus (Dawid et al., 2007). In this study, we analysed a series of co-colonizing isolates to evaluate the impact of the Blp locus on bacteriocin and immunity protein production to determine whether competitive phenotypes of bacteriocin secretion restrict co-colonization. We have identified that TIGR4 can produce functional bacteriocins using overlay assays and a novel inhibition assay. In this study, we analysed the *blp* locus of TIGR4 regarding pheromone type, bacteriocin/immunity content, and function.

First, we explored the role of *blp* locus and bacteriocin/immunity protein secretion in the presence of streptococcal strains. The requirement for bacteriocin and immunity proteins for full inhibitory activity in TIGR4 has not been described previously, and it represents a unique approach to bacterial competition. During work on the *blp* locus, we noted that the invasive TIGR4 serotype had significant antibacterial activity against *S. parasanguinis.* We constructed a TIGR4 *blp* knock-out strain, containing a deletion of the entire *blp* bacteriocin locus. We also determined the role of the *blp* gene locus, required for bacteriocin and immunity proteins production, using the overlay assay. Deletion of the *blp* locus encoding the bacteriocin and immunity proteins resulted in loss of both inhibition and immunity.

Second, we developed a novel inhibition assay using the co-culture of producer and target in liquid culture and assaying for target inhibition by colony counting.

Third, we investigated the growth of *S. pneumoniae* in an ion deficient medium. Many bacteriocins use metal ion transport proteins to attach. These transporters are typically upregulated under conditions of low concentration to their cation cargo. We also studied the effects of ion(s) deficiency on bacteriocin activity and its ability to kill related streptococcal species. There are significant differences in inhibition ability in different depleted media and these are being investigated further. The induction of the Blp bacteriocins depends on quorum sensing. This is dependent on pH of the media, requiring a pH of 8 or more for efficient expression. The effects of different pH have been assayed on TIGR4 and other pneumococcal strain bacteriocin activity – we noted strain variation in pH dependence.

The expression of the Blp locus is also co-regulated with the competence regulator system. We analysed the effect of activation of the competence pathway on bacteriocin expression. The addition of CSP a specific peptide that stimulates competence also increased bacteriocin production.

In addition, while Blp bacteriocin suppresses rival streptococci strains and species, there is an interesting observation of TIGR4 bacteriocin resistance in *S. parasanguinis*. This was observed in a co-culture of TIGR4 and *S. parasanguinis* strain in BHI media, where *S. parasanguinis* was able to grow in the presence of the TIGR4 produced Blp bacteriocin. We hypothesized that this unusual TIGR4 bacteriocin resistance is due to genetic mutations in the *S. parasanguinis* strain, which rendered it non-sensitive to the killer bacteriocin. Alternatively, it is also possible that the bacteriocin-producing TIGR4 strain has a self-resistance gene against self Blp bacteriocin, which has been transferred to *S. parasanguinis* through horizontal gene transfer (HGT).

Finally, we engineered a pneumococcal expression vector to express a putative immunity protein with a His tag. The vector has been successfully transformed into TIGR4. We hypothesised that the gene transfer by transformation procedure would allow bacteria to confer resistance to the wild-type TIGR4 produced bacteriocin. Expression of the immunity protein was determined using immunofluorescence.

CHAPTER 2: Materials and Methods

2.1 Strains and Plasmids

All strains and constructs used in this research are listed in (Table 2.1).

Table 2.1: Streptococci strains used in this research study.

Strains	**Description**	**Source**
S. salivarius	Wild type strain; closely related streptococcal strain	Isolated from patient's oral normal flora
S. anginosus	Wild type strain; closely related streptococcal strain	Isolated from patient's oral normal flora
S. equinus	Wild type strain; closely related streptococcal strain	Isolated from patient's oral normal flora
S. faecalis	Wild type strain; closely related streptococcal strain	Isolated from patient's oral normal flora
S. oralis	Wild type strain; closely related streptococcal strain	Scottish Microbiology Reference Laboratories
S. mutans	Wild type strain; closely related streptococcal strain	Scottish Microbiology Reference Laboratories
S. parasanguinis	Wild type strain; closely related streptococcal strain	Scottish Microbiology Reference Laboratories
ST180		Scottish Microbiology Reference Laboratories
P940	Genetically engineered to contain beta-galactosidase gene	Kindly donated from Dr Suzanne Dawid ((Son et al., 2011)
P4	Reference producer Strain	Kindly donated from Dr Suzanne Dawid ((Son et al., 2011)
P1042	Reference reporter Strain	Kindly donated from Dr Suzanne Dawid ((Son et al., 2011)
TIGR 4	Type strain	Supplied by professor Tim Mitchell
P1104	Genetically engineered to contain beta-galactosidase gene	Kindly donated from Dr Suzanne Dawid ((Son et al., 2011)

Table 2.2: List of plasmids.

Plasmid	Description
pLS1ROM	Includes MCS sequence to facilitate the insertion of the target gene. This vector is characterized by an improved expression from the regulated promoter PM and an increased stability.
pSC-B-amp/kan	Strata Clone Blunt PCR Cloning
pBAV-K1-T5-gfp	downstream a Kpnl promter, contains a-gfp open reading frame.

2.2 Chemicals

2.2.1 Growth Medium

Unless listed otherwise, the chemical reagents used in this thesis were supplied through major suppliers, including Sigma-Aldrich, and the College of Medical, Veterinary and Life Sciences local stores. The culture media used included Todd Hewitt broth with yeast extract media (THY), brain heart infusion (BHI) medium, Luria-Bertani (LB) agar plates, LB liquid medium, and Super Optimal Broth (SOB medium) medium. For sensitivity tests, antibiotics and chromogenic substrates used included erythromycin 10 μg/ ml (Sigma Aldrich), kanamycin (50μg/ml, Sigma Aldrich), streptomycin (50 μg/ml, Sigma Aldrich), and/or X-gal (20μg/ml, Roche).

2.2.2 Enzymes

Restriction endonucleases for DNA manipulation used in this study were obtained from New England Biolabs (NEB). The DNA polymerase used for making polymerase chain reactions (PCRs) applied for cloning and gene knockout was High Fidelity Platinum® Taq DNA Polymerase from Invitrogen. T4 DNA ligase from Thermo Scientific was used for specific ligation reactions.

2.2.3 Bacterial Strains Growth Conditions

Streptococcus strains used in this study are shown in Table 2.1. Streptococci strains used in the research were wild type (clinical isolates) and mutants *S. pneumoniae* and mutants. *S. pneumoniae* TIGR4, TIGR4 knock out, *S. salivarius and S. parasanguinis* were routinely

cultivated in Todd-Hewitt broth (Sigma Aldrich) with 0.5% yeast extract (Sigma Aldrich) (THY media) at 37°C with 5% CO_2. The normal THY medium was treated with chelating agent Chelex-100 (Sigma Aldrich) for at least 8 hours, then adding ions ($CaCl_2$) to a various concentration at 100μM, 200μM, 500μM, and 1000μM, magnesium chloride ($MgCl_2$) at 100μM, 200μM, 300μM, 500μM, manganese sulphate at 2μM, 5μM, 10μM, 50μM, ferric chloride at 2μM, 5μM, 10μM, 50μM, zinc nitrate at final concentration 500μM, and copper nitrate at final concentration at 100μM. To measure the minimum different ion concentration required for each bacterial strain growth, the optical density OD_{600} values were detected with addition of various ion concentrations at different time frame. The initial inoculum concertation of the different bacterial strains was started from OD_{600}=0.2. *S. pneumoniae from* blood agar plates was identified based on α-haemolysis, indicated by greenish zone of haemolysis around colony growth, and the characteristic greyish draughtsman-like colonies. Pneumococcal growth was confirmed by addition of optochin-resistant disc (Sigma Aldrich) and bile solubility testing. Plates were incubated at 37°C with 5% CO_2, and a zone of inhibition was considered optochin sensitive.

2.3 Bacterial Stocks Storage

Bacterial strains used in this study was maintained by glycerol stocks at -80°C. Glycerol stocks were prepared from an overnight culture growing from a single colony in appropriate growth media, supplemented with antibiotics as needed. The final stocks were stored in aliquots of 1 ml with the addition of 15% sterile glycerol. TIGR4 and other streptococcal strains were routinely grown at 37°C, with 5% CO_2 in Todd Hewitt broth with yeast extract media (THY) or brain heart infusion (BHI) medium for overnight culture. Based on transformed plasmids or chromosomal markers, antibiotics were added when appropriate.

To make competent cells, *E. coli* cells were routinely grown 37°C using Luria-Bertani (LB) agar plates, LB liquid medium, or SOB medium with the addition of appropriate antibiotic

where needed. Liquid cultures were grown overnight with shaking at 225 or 250 rpm. Ampicillin (100μg/ml) and kanamycin (50μg/ml) antibiotics (Sigma-Aldrich) were used where appropriate for each plasmid used. For culture in liquid medium, strains are inoculated into Todd-Hewitt broth supplemented with 0.5% fresh yeast extract (Sigma Aldrich), pH 7.8. For growth on culture agar plate, blood agar base supplemented with 5% defibrinated horse serum is used.

Where indicated, catalase and 5-bromo-4-chloro-3-indolyl-β-D- galactopyranoside (X-Gal) are added to a concentration of 3000-4000 U (Sigma-Aldrich) and 40 mg/ml, respectively. Where applicable, media are supplemented with erythromycin (Em) (Sigma Aldrich) or Kanamycin (Km) or streptomycin (Sm) (Sigma Aldrich) at concentration of 6 μg/ml, 50 μg/ml and 50 μg/ml, respectively.

2.4 Streptococcal Culture

A sterile cotton swab is used with a streak isolate of *Streptococci* grown on a blood agar plate (BAP) and incubated at 37°C with 5 % CO_2 overnight. Single colony was then re-streaked on two plates: 1) across the entirety of a fresh plate and 2) using the three-phase streak method. The plates were incubated at 37°C with 5% CO_2 overnight. Uniform colonies appearing on the three-phase plate are streaked using a sterile cotton swab and inoculated bacterium from the lawn of the other plate in 5mL of TSB. Upon reaching OD_{600} =0.5, 800μL the culture is frozen down with 200μL 80% glycerol and stored at -80°C.

2.5 Gram Staining

A single colony was spread on a glass microscope slide. The primary stain (crystal violet) is applied to the smear. Gram's iodine (mordant) is added, before rapid decolorization with acetone. Counterstaining with safranin-red. Visual confirmation was achieved using microscopic examination at (100X), Gram-positive streptococci were indicated by purple colour and observed shape is round (cocci) appearing in clusters or in chains.

2.6 Catalase Test

To conduct catalase test, the isolates to be tested (Table 1) are grown for 18-24 hours on BAP at 37°C with 5% CO_2. A disposable loop is used to remove a colony from overnight growth on BAP to a glass slide. Care is taken to prevent transfer of blood agar to the slide to avoid a false-positive catalase reaction. Catalase negative bacteria are indicated by the absence of bubbling after the addition of 1 ml of 3% H_2O_2.

2.7 Optochin (OP) Sensitivity Test

Strains to be tested (Table 1) are obtained from overnight colonies grown on BAP at 37°C with 5% CO_2. A OP disk is placed within the streaked area of the plate and incubated overnight at 35-37°C with ~5% CO_2. Observation for growth on the BAP near the optochin disk is observed and the zone of inhibition measured. A zone of inhibition of 16 mm determined using a 6 mm, 5 µg disk is observed indicating sensitivity, and presumptive identification of pneumococci.

2.8 Bile Solubility Test

The sodium deoxycholate (bile) solubility test is conducted to distinguish presence of *S. pneumoniae* from other alpha-haemolytic streptococci (Table 1). *S. pneumoniae* is bile soluble while other alpha-haemolytic streptococci are resistant. About 2% of Sodium deoxycholate in water is used to check lysis of the pneumococcal cell wall. Bacterial growth from the overnight BAP is added to 1.0 ml of 0.85% saline, achieving turbidity ranging from 0.5 McFarland standard. The cell suspension is divided equally into 2 tubes each consisting of 0.5 ml. Approximately 0.5 ml of 2% sodium deoxycholate is added to one tube, and 0.5 ml of 0.85% saline added to another tube followed by thorough mixing. The mixtures are incubated at 35-37°C in CO_2 and vortexed. After 10 minutes, the tubes are observed for any clearance indicating bile solubility, if no solubility is indicated the tubes are incubated for 2 hours at 37°C in CO_2. Clearing of turbidity in the bile tube (but not in the saline control tube) show a positive bile solubility test.

2.9 Antibiotic Susceptibility Test

The chloramphenicol susceptibility method was performed to assess *S. pneumoniae* strains have correct antibiotic resistance. To perform antibiotic susceptibility test chloramphenicol (2μg/ml) was mixed with blood agar medium. The mixture was then poured in sterile round petri dish, and allowed to solidify at room temperature. Reporter strains (P1042, P940, P1216, and P1104) were inoculated onto the plates and incubated overnight at 37 °C with 5% CO_2. Next day, strains are examined for growth on plates which indicate resistance for chloramphenicol.

2.10 Bacteriocin Induction

Cells were grown to OD_{600} 0f 0.4 in 5 ml THY medium. The cultures were then supplemented with either competence stimulating peptide (CSP-1) or (CSP-2) at 100 ng/ml, and incubation continued for another 2-3 hours. The cultures were then split by centrifugation at 16, 000 rpm and the supernatants were recovered; the supernatant should contain the bacteriocins as well as the BlpC pheromone.

The antibiotic susceptibility method was used to induce a stress response and tested to induce bacteriocin action, and was performed to determine the effect of addition of Streptomycin (Sigma Aldrich) to a final concentration of 6μg/ml. The effect of Streptomycin was then assessed on both the plate inhibition assay as well as the reporter gene induction.

2.11 Overlay Assay

Overlay assays were performed as previously described (Maricic & Dawid, 2017). Strains to be tested for bacteriocin production were streaked on a 5% sheep blood agar plate from -80 °C freezer stocks. The use of blood plates greatly enhances the reproducibility of the assay. Blood agar plates were incubated overnight at 37 °C with 5% CO_2. Following incubation of the producer strain, tryptic soy agar (TSA) plates were prepared by pipetting 100 microliters of catalase (3, 000 Units) onto plates containing 25 ml of TSA and the catalase solution was spread

using a sterile plastic spreader. Plates were allowed to dry for 10 minutes under class 2 safety cabinet. Strains to be assayed for inhibitory activity were stabbed into the dried TSA plate. More than one producer strain can be spiked into a single TSA plate; however, it is crucial to space out the stabs so that readings do not overlap. Plates were incubated for 8 hours at 5% CO_2 to allow for outgrowth. The reporter strains were grown to an optical density (OD) of 0.5 and 200 microliters were added, just prior to overlay application, to a mixture of 100 microliters of catalase, 5 ml of THY media, 50 microliters of X-gal (40 mg/ml), and 3 ml of molten TSA were added. X-gal was used as a chromogenic substrate for β-galactosidase where it marks site of enzymatic activity by forming dark blue precipitate. The addition of catalase to both the producer and reporter strains is very important to remove the inhibitory effects of pneumococcal H_2O_2 production, which might interfere with the overlay assay results. The overlay mixture allowed to equilibrate at room temperature prior to addition of molten TSA. Then, the mixture was carefully poured using a dispenser over the top of the stabbed TSA plates, and incubated overnight at 37 °C in 5% CO_2 incubator. A blue clear zone around the stabs indicates inhibitory activity.

2.12 Overlay Assay (Modified test)

For our needs, we had to adapt a test based on the overlay assay described above to determine the activity of bacteriocin production. The assay is based on a dual-layer TSA agar plate system in a 12-cm petri dish. The bottom layer consisted of 25 ml of TSA agar on which a hole was made using a sterile glass pipette. 3000 U of catalase were spread onto the agar and allowed to dry for 10 minutes. The bacteriocin- producing test strain was diluted 1:10 following an overnight culture at 37 °C with 5% CO_2, and inoculated into the hole. The plates allowed to grow for further 6 hours at 37 °C with 5% CO_2. The top layer consisted of 200 microliters reporter strain, 100 microliters of 3000-4000 U catalase, 5 ml of THY media, 50 microliters of

X-gal (40 mg/ml), and 3 ml of molten TSA. The plates were incubated for overnight at 37 °C with 5% CO_2. Next day, plates were examined for antagonistic inhibition activity.

2.13 Synthesis of BlpCR6 bacteriocin inducer

The BlpCR6 bacteriocin inducer was commercially synthesized (ThermoFisher). The BlpCR6 bacteriocin inducer has cleavage sites between the A18 and L19 residues, at its C terminus (Ratner et al., 2022). The synthetic inducer was formed with the sequence [NH2] GWWEELLHETILSKFKITKALELPIQL [COOH.acetate]. The synthetic inducer was used to detect inhibitory effect of the BlpC inducer peptide alone at 500ng/ml in a inhibition assay targeting several reporter strains.

2.14 Molecular method

2.14.1 Species-specific PCR assays

PCR detection of*S. pneumoniae* is achieved by amplifying several potential gene targets. In this study, the assays used were developed and validated from bacterial isolates (Table 2.7.1.1). Both real-time and conventional PCR assays have been developed for *S. pneumoniae*, with target genes being pneumococcal surface adhesion (*psaA*), autolysin (*lytA*), and pneumolysin (*ply*). Nonetheless, false-positive findings with *ply*-based PCR have been reported when applied to specimens obtained from the upper respiratory tract. A potential explanation for such false positives relates to non-pneumococcal alpha-hemolytic streptococci such as *Streptococcus oralis* and *S. mitis* group that contain the *ply* gene.

To address the problem, PCR detection assay for *S. pneumoniae* is based on autolysin gene (*lyt*A) because it is highly conserved. Also, the autolysin gene (*lyt*A) best helps in distinguishing *S. pneumoniae* from similar species such as *S. pseudopneumoniae, S. oralis,* and *S. mitis.* The real-time PCR assay *lytA* probes and primers are largely reliable for detection of *S. pneumoniae*. However, due to recombination events that occur between pneumococci and

closely related streptococci, it is possible to obtain rare false-negatives or false-positives for any real-time assay during pneumococcal identification.

2.14.2 Genetic Pneumococcal differentiation

S. pneumoniae shares 99% 16S ribosomal RNA sequence similarity with *S. mitis* and *S. pseudopneumoniae* (Kawamura *et al.* 1995). However, recent studies suggest that internal species-specific signature sequences of the 16S ribosomal RNA gene may distinguish these species (El Aila et al. 2010). DNA-based methods include sequence analysis of the housekeeping genes (Kawamura *et al.* 1995). Other targets for species differentiation include Spn9802, Spn9828, cpsA, recA, and psaA (El Aila et al. 2010).

2.14.3 Genomic DNA extraction

All DNA samples was extracted using theDNeasy® Blood and Tissue kit supplied by Qiagen. Protocols were followed as indicated by manufacturer's instructions. *S. pneumoniae* strains were grown in 10 ml brain heart infusion (BHI) media at 37 °C with 5% CO_2 to OD_{600nm} 0.5. The culture was centrifuged at 5000xg (7500 rpm) for 10 minutes to harvest the cells. The supernatant was removed. To begin the extraction procedure the pellet was re-suspended in 180 µl enzymatic lysis buffer (20mM Tris.Cl pH 8.0, 2mM sodium EDTA, 1.2% Triton®X-100, 20mg/ml lysozyme) and incubated for at least 30 minutes at 37 °C. subsequently 25 µl proteinase K and 200 µl buffer AL (provided by the kit), without ethanol was added and mixed by vortex. Then the suspension was incubated at 56 °C for another 30 minutes. 200µl ethanol (96-100%) was then added to the sample, and mixed thoroughly by vortex. The mixture was then transferred into the DNeasy Mini Spin Column, and placed in a 2ml collection tube. Then, it was centrifuged at > 6000 x g (8000 rpm) for one minute. The flow-through then was discarded. The DNeasy Mini Spin column was placed in a new 2 ml collection tube (provided by the kit). 500µl buffer AW1 were added, and further centrifuged for 1 minute at >6000 x g (8000 rpm).

The flow through and collection tube were then discarded. The DNeasy Mini Spin column was then placed in a new 2 ml collection tube (provided by the kit). 500µl of buffer AW2 were added and centrifuged for 3 minutes at 20,000 x g (14,000 rpm) to dry the DNeasy membrane. The flow through and collection tube were discarded. The Dneasy Mini Spin column was placed in a sterile 1.5ml microcentrifuge tube. 200µl buffer AE was transferred directly onto the DNeasy membrane. The column was incubated at room temperature for one minute, and then centrifuged for one minute at >6000 x g (8000 rpm) to elute the DNA. The DNA concentration was then measured using the nano drop device.

2.14.4 Extracting genomic DNA from clinical isolates and specimens

PCR experiment requires a successful DNA template extraction procedure. The objective of DNA extraction is to lyse the bacterial cells in the specimens to increase the yield and quality of bacterial DNA while removing any PCR inhibitors (such as proteins, salts), dissolve the DNA in a buffer compatible with the enzymes used in the following step, and concentrate the DNA at the same time. It is crucial to choose a DNA extraction technique that will yield enough DNA for PCR detection (dependent on the assay's lower limit of detection) while also filtering possible PCR inhibitors. The kind and size of the material, the nucleic acid desired (RNA or DNA), the concentration of the target DNA contained in the specimen, the presence of contaminants that might act as PCR inhibitors, the equipment and facilities present, and safety regulations are things to take into account. In general, techniques with fewer stages reduce the possibility of contamination and DNA loss. Along with biochemical and physical techniques, there are commercially accessible techniques for both cell lysis and purification, such as those utilizing silicon membrane, spin columns, and magnetic beads. As long as the manufacturer's guidelines are strictly adhered to, these methods are considered to typically yield satisfactory results.

During the transformation of *S. pneumoniae* for genomic extraction, colonies of *S. pneumoniae* are grown in 10ml BHI with 1mM $CaCl_2$ until OD_{600} of 0.1 is reached. Aliquots of 1 ml of the culture are prepared, and 100ng/ml competence stimulating peptide 1 (CSP-1, company) for ST-180, or CSP-2 for TIGR4, is added to induce uptake of extracellular DNA. Samples are incubated at 37 °C with 5% CO_2 for 15 minutes. Samples are further incubated at °C with 5% CO_2 for 75 minutes following the addition of 500ng DNA. Samples finally plated on blood agar plate containing 0.5µg kanamycin or another antibiotic where required.

2.14.5 Purification of DNA

2.14.5.1 Bacterial Cell Lysis.

Gram-positive cell suspensions (i.e., *S. pneumoniae*) were digested using digestion buffer (75 U/ml mutanolysin and 0.04 g/ml lysozyme in TE buffer). The solution is prepared fresh 15 minutes before use. 100 µl of digestion buffer is added to each microcentrifuge tube. Then, 200 µl of bacterial cell suspension is added to each microcentrifuge tube, vortexed, and incubated at 37°C for 1 hour. To each microcentrifuge tube, 200 µl of cell lysis buffer is added, followed 20 µl of Proteinase K (20 mg/ml) at 65 °C (Sigma Aldrich). Each tube is then inverted until the phases are completely mixed. The solution is incubated at 37°C for 30-60 minutes. The DNA is then purified in preparation for PCR reactions.

2.14.5.2 Precipitation of DNA.

DNA is precipitated by 95 % ethanol by adding a 0.1 volume of 3.0 M sodium acetate (pH 5.5) to the aqueous phase, followed by 2 volumes of ethanol. The mixture is incubated at -20°C overnight before centrifuging at 16,000 x g for 30 min at 4°C. The precipitated DNA is recovered by centrifuging the tube at 16,000 x g for 15 minutes at 4°C. The aqueous phase is removed carefully. To remove excess traces and salts of chloroform and phenol, 2 volumes of the original sample are added (75% (v/v) ethanol) and left at room temperature for 5-10 minutes. The product is centrifuged at 16,000 x g for 5 minutes. The DNA pellet is dried in a

50°C oven for 5 minutes. The dried DNA is dissolved in sterile Tris buffer (10mM Tris-HCl, pH 8.0) and stored at -20°C for long-term storage or 4°C for subsequent manipulation.General DNA manipulations were performed using standard protocols (Evans 1990; Bartel 1991).

2.14.5.3 Storage of DNA.

Extracted and purified DNA is stored in a Tris buffer (10 mM Tris-HCl, pH 8.0). While distilled water could be used, it is avoided since the specimens may experience degradation due to acid hydrolysis. Long-term DNA storage is achieved at -20°C.

2.14.6 Standardized Polymerase Chain Reaction (PCR)

Each PCR reaction contains 50 µl master mix consisting of 10µl of 10X reaction buffer (NEB builder), 1µl dNTP mix of 10 mM each (NEB builder), 5µl of each oligonucleotide primer (IDT), 1µl of DNA Phusion™ High-Fidelity DNA Polymerase (NEB), 1µl of the sample, and 27µl of RNase-free distilled water (Invitrogen). Table 2.3 lists primers used for conventional PCR which is performed in this research study and the amplification extension time for each primer pair. Despite the differences between the melting temperature (Tm) and extension time, all PCR reactions performed had an initial denaturation step at 95°C, followed by 30 cycles of amplification. Amplification included 30 seconds of denaturation at 95°C, 30 seconds of annealing at various temperatures (per the primer Tm), and a final extension at 72°C for 2 minutes.

PCR reactions were conducted using the Techne Thermal Cycler (Jencons-pls). The primers used in PCR reactions are listed in (Table 2.3) were supplied by Integrated DNA Technologies (IDT). A final concentration of 100 µM of re-suspended lyophilized primers using RNase free water was prepared. PCR products were amplified using DNA polymerase Phusion HIFI from NEB. PCR master mix was prepared by adding the following ingredients: 10 µl 5X High Fidelity PCR Buffer, 1µl dNTP , 10 µM forward primer, 10 µM reverse primer, 1 µl Template DNA, 1 µl DNA polymerase, and RNAse free water up to 50 µl. Parameters for PCR yields

<12kb were; initial denaturation at 95°C for 2 minutes, 30X cycles of denaturing at 95°C for 30 seconds, annealing at variable temperatures depending on primer melting temperature (T_m) for 30 seconds, and extension at 72°C for 1 minute/kb, then a final extension at 72°C for 3 minutes. PCR products were held in the thermocycler at 4°C and then stored at -20°C.

Table 2.3: Conventional PCR primers used in this research study.

Number	Name	Primer sequence in 5'-3' orientation
1	BlpC Forward	CTA GAC TCG AGA TAA AAA ACA AGA CCG AG
2	BlpC Reverse	TCG AAG GAT CCG AGA ATC ATA ACA TGG AT
3	BlpCS Forward	CTA GAC TCG AGT CTT GTT TCT CGG TCT TGT T
4	BlpCS Reverse	TCG AAG GAT CCG GTG GAG AAT GGT GGG AAG
5	BlpCSN-Forward	CTA GAC TCG AGT CTT GTT TCT CGG TCT TGT
6	BlpCSN- Reverse	TCG AAG GAT CCA AGA TTA CAG GTG GAG AAT GG
7	SJ2-F	CTT CCT AGT TTT AAG GAT CGA TCC GTT TG
8	SJ2-R	AAA GCA TAT GTT ATG CTT TTG GAC GTT TAG
9	TBlpDS-F	AAA AGC ATA ACA TAT GCT TTA CAA CAG TTG GC
10	TBlpDS-R	TTG CCT TGG CTT CCA AGG
11	TBlpUS-R	CGA TCC TTA AAA CTA GGA AGC TAG CCG C
12	TBlpUS-F	AAG GGA ATT TCA AAC AGC G
13	MN immunity insert US	ATT GAA TTG TTT TGT CAT ATT ATT TCT AAT CTA TG
14	MN immunity insert DS	GAT GTT AAT TCA ATA GTC TTT TG
15	MN immunity US	ATG CGC AAG CTT AAG GCT ATA TTC TTT ATT ATT
16	MN immunity DS	ATG CGC CTG CAG TTT GTA TCC ATA TAG TGT
17	US2F	TGA AGA TAA AGC CTG TAA
18	DS2R	AAG TTG ATT GGT TCT AGG
19	BlpAreverse	ATG ACT TCT TAT AAA CGT ACA
20	BlpAforward	TTA TGG TAA CGT CTC TGA TA
21	pBAV DS	ATG CGC CTG CAG GCT AGC AAG CTT ATG GTG ATG GTG ATG GTG CAT CTA GTA TTT CTC CTC TTT C
22	pBAV US	ATG CGC GAA TTC GCG GCC GCT TCT AGA GG
23	Blp-upstream-fwd	AAG GGA ATT TCA AAC AGC G
24	Blp-upstream-rev	CGA TCC TTA AAC TAG GAA GCT AGC CGC
25	SJ-fwd	CTT CCT AGT TTT AAG GAT CGA TCC GTT TG
26	SJ-rev	AAA GCA TAT GTT ATG CTT TTG GAC GTT TAG
27	Blp-downstream-fwd	AAA AGC ATA ACA TAT GCT TTA CAA CAG TTG GC
28	Blp-downstream-rev	TTG CCT TGG CTT CCA AGG
29	rpslUP	GGA CTA GTA GAA GTA GTT GGC
30	rpslDOWN	CGG AAG TGT GCG AAT GCA CGG
31	New Upstream Fwd	ACG CAG GTC AAG GGA ATT TCA AAC AGC G
32	New Downstream rev	CCC GCC ATT TTG CCT TGG CTT CCA AGG
33	RPSL-up2	TTC TGT TGG GTT CGT T
34	RPSL-DS2	CCT TGC ATC CTT ACC GTT

2.14.7 Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to separate genomic and plasmid DNA. Verification of each PCR reactions and analytical restriction digests of clones were performed using 1% agarose gel. Gels were prepared with 1X Tris-acetate-EDTA (TAE) buffer and 10 µl SybrSafe® gel stain in DMSO (Invitrogen). For safe and convenient loading and tracking of DNA samples, a 1:5 volume of 5X loading dye (bromophenol blue, xylene cyanol, and orange G) was added to samples prior to loading for reliable estimation of DNA migration on the gel. Generally, gels with loading samples were run at 100 volts (V) for ~60 minutes, or until the bands of ladder could be visualised easily. A marker of 1 kb size or 100 bp (NEB) was used as a ladder to compare the sample band size. DNA visualisation was achieved using uviPRO gold Uvitec transilluminator (Uvitec). Electrophoresis gel with 1% agarose was prepared by adding 1 gram of agarose (Biorad) to 100ml of 1 x TBE buffer. The mixture was heated in a microwave oven to boiling to melt the agarose powder. 5µl SYBR™ Safe DNA Gel Stain (Invitrogen) were added to the mixture and the gel was allowed to solidify at room temperature.

2.15 DNA Cloning into Plasmid Vectors.

The assembled product of vectors and inserts were then used for the expression or the deletion of desired genes in $TIGR_4$. This were achieved by cloning the target genes into the PBAV plasmid, downstream of a Kpnl promoter (Topp S et al. 2010). The kanamycin resistant plasmid contains a-gfp open reading frame.

The StrataClone ™ Blunt PCR cloning kit was used to clone blunt-ended PCR product of the *MNO* insertion into the plasmid. The StrataClone ™ Blunt PCR cloning kit was used per manufacturer's instructions. Briefly, each StrataClone ™ Blunt PCR cloning reaction mixture was prepared by mixing the following components gently by repeated pipetting: 3µl of StrataClone ™ cloning buffer, 2µl (50 ng) of blunt-ended PCR product or StrataClone ™ Blunt

control insert, and 1µl StrataClone ™ vector mix. Blunt-ended PCR products were then ligated between vector arms in a 5 minutes' reaction at room temperature, and then placed on ice.

2.16 Cloning and Expression of BlpMNO

Primers used to amplify the immunity protein insert (BlpMNO) by PCR are listed in Table 3.1. Phusion high-fidelity taq polymerase enzyme was used to amplify each open reading frame. PCR products were purified, digested with EcoR1 and PstI (New England Biolabs) and ligated to PBAV at EcoR-1 and PstI restriction sites. The ligated product was transformed into electrocompetent *E. coli* XL blue 1 (Aglient) and selected on LB agar plates supplemented with 100 µg/mL ampicillin (Sigma Aldrich) or 50 µg/mL kanamycin (Sigma Aldrich).

2.17 Restriction Digest

Restriction endonucleases (High Fidelity (HF®) and buffers for DNA manipulations were acquired from New England Biolabs (NEB). Restriction digests were prepared in a final volume of 20µl. For analytical purposes, samples were prepared to meet the following criteria, 1-3µg of PCR product or plasmid DNA was digested with a 1X appropriate restriction buffer and 10 units of desired restriction enzyme(s). The digests then were incubated in water bath at 37°C for 1-2 hours. Finally, the digests were purified after digestion using Qiagen PCR purification kit.

2.18 Gel Extraction and PCR Purification

DNA fragments used for cloning were purified prior the ligation step. The correspondent band size of DNA fragments, intended to be obtained from a preparative plasmid digest, was extracted from the agarose gel by using QIAquick® Gel Extraction Kit (Qiagen) per manufacturer's instructions. PCR products were then purified using the QIAquick® PCR Purification Kit (Qiagen) directly from the PCR reaction mixture. Following purification, DNA

concentrations were measured using the Nanodrop 1000 spectrophotometer (Thermo Scientific).

2.19 Ligation Reactions

Ligation reactions were conducted using 50-150 ng of digested plasmid backbone. The insert added at a 1:2 molar ratio (plasmid: insert). Ligations reactions consists of 10X DNA ligase buffer (1X final concentration) and 1 Weiss u/µl T4 DNA ligase (1 u in total, Thermo Scientific), a total volume of 20µl of ligation mixture was prepared using RNAse free water. Reactions were incubated at room temperature for 1 h, then 10 min at 65°C for inactivation of restriction enzyme(s).

2.20 NEBase Changer Reaction

The NEBase changer reaction was done according to manufacturer's instructions for site-directed mutagenesis. First, exponential amplification PCR was done to amplify the PCR product. Then, kinase, ligase, and Dpnl digestion was done for 5 minutes at room temperature. The mixture was then transformed into TIGR4 wild type strain.

2.21 Construction of the Sweet Janus (SJ) Cassette for the BlpMNO Gene Knock Out

To construct a SJ cassette between DNA sequences corresponding to the genes that flank the Blp locus, two DNA fragments, Upstream and Downstream, were amplified by PCR using genomic DNA of TIGR4 as template. The DNA fragment upstream contains 662bp of the BlpMNO upstream sequence, and 81-bp of the constitutive promoter sequence in Janus cassette. DNA fragment upstream and downstream contains Kan-rpsl^{+}sequence of the Janus cassette and 1081-bp of the BlpMNO downstream sequence. To assemble the DNA fragments upstream and downstream into SJ, primers were designed to contain 25-bp overlap region at the junction's regions and the net PCR products ligated using the Gibson Assembly Kit (New England BioLabs, NEB builder) per manufacturer's instructions. The ligated DNA product was used as a template for PCR reaction using the primers US and DS. The amplified DNA product

was used to transform TIGR4 with selection to Kanamycin resistance to create TIGR4-Blp locus knock out.

2.22 Transformation of *S. parasanguinis*

Cells were cultivated by adding 1% overnight pre-culture to fresh BHI medium. Cells were grown at 37°C with 5% CO_2 without agitation to a cell density equivalent to OD_{660} of 0.5. Glycine was then added to a final concentration of 10% and incubated for another one hour at 37°C with 5% CO_2. Cells were centrifuged at 14,000 rpm for 5 minutes. Cells were then washed twice in an ice-cold washing solution (5mM potassium phosphate (pH 4.5), 0.4M sorbitol, 10% glycerol). In electroporation solution (5mM potassium phosphate (pH4.5), 0.4M sorbitol, 10% glycerol), and to produce electrocompetent cells, cells were diluted 50 folds and stored at -80°C following freezing in an ethanol-dry ice bath. These cells were thawed on ice, and 1µg plasmid DNA were added to a 100µl of electrocompetent cells. The mixture was homogenized by gently mixing with pipette for several times. The mixture was then transferred into a pre-chilled cuvette. The moisture from the cuvette was wiped, and the cuvette was inserted into the electroporation device. Electroporation was done at voltage 1600V and time constant 5ms. 1ml of ice-cold outgrowth medium was added to the mixture and further incubated for 3 hours at 37°C with 5% CO_2. The aliquot was inoculated onto selective agar plate and further incubated for 2-3 days at 37°C with 5% CO_2.

2.23 Liquid Inhibition Assay (Tube Method)

A liquid inhibition assay was used to determine the effectiveness of a compound or substance at inhibition microorganisms in liquid form. The method was developed in this study to assess the inhibition of producer strains on reporter strains. TIGR4, TIGR4 knock out and *S. parasanguinis* each were growing in 10ml BHI or, 10 ml of BHI de-cation media, overnight at 37°C with 5% CO_2. Aliquots of 1 ml of each culture were taken. 1ml of the producer strain

were mixed with 1ml of the reporter strain, with or without the addition of CSP2 (250 ng/ml) for TIGR4, and six tubes were prepared. The tubes were incubated 37°C with 5% CO_2 on one-hour intervals up to six hours. Serial dilution of a mixture of 1 ml of producer and 1ml of reporter strain was done up to 10^6. Blood agar plates were prepared with 50μg/ml Kanamycin or 2μg/ml optochin. Three drops were placed on the blood agar plate containing the desired antibiotic and incubated at 37°C with 5% CO_2 for overnight. Next day colonies were examined for growth which indicate resistance to the producer bacteriocin production.

2.24 Bacterial strain identification VITEK 2

Bacterial strains were identified using VITEK 2. Briefly, bacterial suspensions, OD 0.5, were loaded on ID card. The ID cards were then run in the VITEK2 device and compared to a set of chemical reactions to produce the ID of the bacteria.

CHAPTER 3: The Role of Blp Locus in *S. PNEUMONIAE* TIGR4 Bacteriocin Production

3.1 Introduction

All strains of *S. pneumoniae* tested to date express some version of the *blp* locus. Therefore, the *blp* locus is widespread and well-conserved in all strains of pneumococcus. However, this locus is characterised by significant genetic diversity, which results in different amounts of cognate immunity peptides and bacteriocins produced by various strains (Dawid et al., 2007; Lux et al., 2007). Figure 3.1 shows the *blp* locus of TIGR4. The *blp* locus conserves genes that encode a typical two-component regulatory system (BlpR and BlpH), a peptide pheromone involved in quorum sensing (BlpC), various bacteriocins and immunity proteins located in the bacteriocin-immunity region (BIR), and an ABC transporter complex (BlpAB). All these components are required for the processing and secretion of the BlpC and the bacteriocin peptides. The *blp* locus of TIGR4 clearly shows that all genes are well-conserved and therefore, should produce bacteriocins. A study by Dawid et al. (2007) demonstrated that the growth of *S. pneumoniae* TIGR4 (virulent serotype 4) was inhibited by *S. pneumoniae* of serotype 6A strain. This finding suggested that either TIGR4 strain did not produce any bacteriocin or produced lower amounts of bacteriocins that were effectively countered by copious amounts produced by type 6A strain, hence the inhibition. Alternatively, the inhibited TIGR4 strain might have expressed insufficient immunity protein to protect itself against the type 6A BlpMN bacteriocins (Dawid et al., 2007). However, these hypotheses need to be tested.

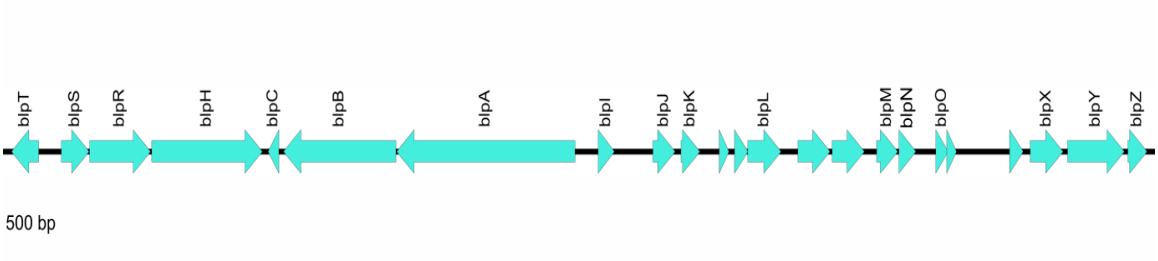

Figure 3-1: Blp locus of TIGR4 showing all the conserved genes that encode a typical two-component regulatory system (BlpR and BlpH).

A previous study demonstrated variations in the integrity of BlpA, the BlpC specificity of BlpH, and the specific content of bacteriocin and immunity proteins. The interplay between these components finally determines bacteriocin production and eventual competition between different streptococcal strains (Son et al., 2011). The highly conserved 4-bp insertion in *blpA*, for example, can be found in nearly half of the strains studied. This insertion results in a frameshift mutation that renders the secretion apparatus non-functional. Strains with a non-functional *blpA* gene could not secrete bacteriocins but are still able to respond to exogenous pheromone because they possess a preserved two-component regulatory system allowing them for the induction of functional immunity genes (Dawid et al., 2007). These strains, called "cheater", were presumably selected because they avoid the energetic cost of bacteriocin secretion. Therefore, the present study aimed to explore the function of the *blp* locus in TIGR4 to clarify the inconclusive findings by Dawid et al. (2007) as to whether it can make active bacteriocins.

3.2 Bacterial Growth Phase

Bacterial growth refers to the proliferation of bacterium into two daughter cells through binary fission. The resultant daughter cells are usually daughter genetically identical to the original cell. When the surviving daughter cells have a higher number than the unity on average, here is an exponential growth of the bacterial population. This is illustrated in Figure 3.2.

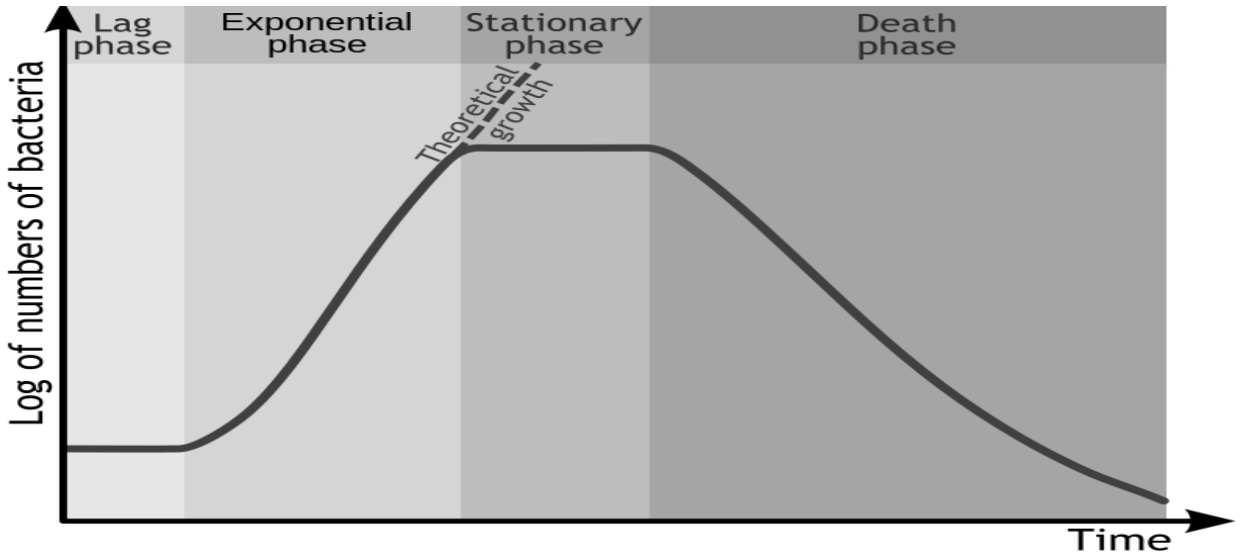

Figure 3-2: The figure shows the Bacterial growth curve\Kinetic Curve. Bacterial cultures go through various growth stages, which include the Lag phase, which is the initial stage of growth, where the bacteria are adapting to their new surroundings and producing the necessary enzymes and proteins for growth. The population of bacteria remains relatively unchanged during this phase. The other phase is the Log phase, which is characterized by a rapid increase in the number of bacteria due to active cell division and growth. The population growth follows a logarithmic pattern. The next phase is the Stationary phase, where the rate of cell growth is balanced with the rate of cell death, resulting in a stable population of bacteria. This phase is typically reached when the available nutrients are exhausted or when the bacteria are under stress. The final phase is the Death phase, in which the number of bacteria begins to decrease as cells die off. This phase is often a result of exhausted resources or harsh conditions. The growth phase of a bacterial culture can be impacted by various factors such as nutrient availability, temperature, pH, and presence of other microorganisms. Understanding these phases is crucial for various applications like production of antibiotics and other industrial products as well as for understanding bacterial growth and behaviour.

3.3 Results

3.3.1 Bacteriocin activity in S. pneumoniae

The entire TIGR4 blp locus was deleted using the Sweet Janaus (SJ) cassette construct. The SJ cassette was constructed by inserting the sacB coding sequence between the promoter region and the start codon of the Kanamycin resistance gene in the SJ (Figure 3.3). Briefly, a PCR product was amplified using primers YL229 and YL236 following Gibson assembly of three PCR products amplified using primer pairs Upstream (US) and Downstream (DS) on SJ cassette template. Next, the SJ cassette was transformed into TIGR4 strain to replace the *blp* locus and to create TIGR4 knockout strain.

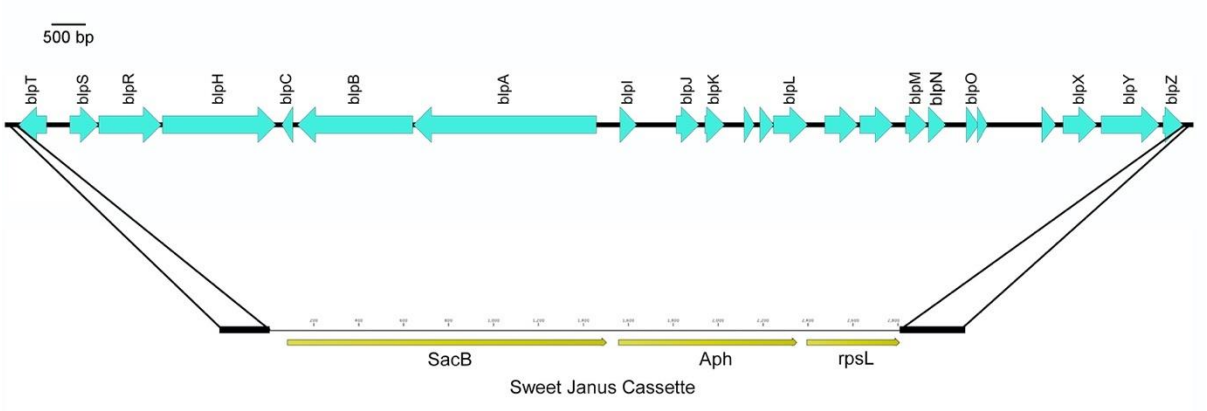

Figure 3-3: Construction of Sweet Janus (SJ) cassette in the blp locus of TIGR4 strain. The figure represents the cassette element and the blp gene flanking the blpMNO locus. rpcL represents the Smr mutant rpsL copy in the genomic region outside the Janus or SJ cassette. The PCR products were assembled into the SJ by using Gibson Assembly and the SJ was used to transform the TIGR4 strain to construct the TIGR4 knockout strain.

First, the ability of various strains to produce the inducer peptide, BlpC, was assayed using reporter strains for the various pheromone pherotypes. The reporter gene constructs used are exactly as described by Son et al. (2011). Briefly, three strains were constructed with lacZ reporter genes fused to the BIR promoter in strains responsive to BlpC types P164 ($BlpC_{164}$,), R6 ($BlpC_{R6}$), and 6A ($BlpC_{6A}$). Importantly, the blpA gene was disrupted in each reporter to make sure that they could only respond to the exogenous introduction of the BlpC. Two additional reporters for T4 and T155 were kindly donated by Kochan and Dawid (2013) and all are listed in Table 3.1. The isolates were inoculated into TSA plates 6 h before the application of an overlay of soft agar containing the BlpC-specific reporter strain and X-Gal (Son et al., 2011), as described in the Methods. Isolates secreting pheromone were detected based on the production of beta-galactosidase resulting in cleavage of the X-gal indicator to produce a blue colouration surrounding the spiked growth. Using this strategy, TIGR4, ST180, and P4 producer strains were identified to have active BlpC pheromone secretion as listed in Table 3.2 and Figure 3.4, consistent with a Blp locus that was functional under *in vitro* conditions tested. The stabbed strains, P4, ST180 and TIGR4, are able to secrete pheromone that interacts with the histidine kinase of the overlay assay, P1042, P980 and TIGR4KO, to

produce blue colour as seen through the breakdown of X-gal as shown in Figure 3.4. If the pheromone is not able to activate transcription of the *blp* locus in the reporter strain, TIGR4 against P1104, or if the stabbed strain does not secrete pheromone, breakdown of X-gal will not occur.

Table 3.1: Reporters for five BlpC types.

BlpC Type	Pre-Protein	Active Peptide
P164	MDKKQNLTSFQELTTTELNQIIGG	GWWEDFLYRFNIIEQKNTKGFYQPIQL
R6	MDKKQNLTSFQELTTTELNQITGG	GWWEELLHETILSKFKITKALELPIQL
6A	MDKKQNLTSFQELTTTELNQITGG	GLWEDILYSLNIIKHNNTKGLHHPIQL
T4	MDKKQNLTSFQELTTTELNQITGG	GLWEDLLYNINRYAHYIT
P155	MDKNQNLTSFQELTTTELNQITGG	GWWEDFLYRFYVVEQSNTKGSNQPVQL

Table 3.2: Streptococcal strains tested by overlay assay with the addition of 50 µl of X-gal (40 mg/ml).

Producer strains	Reporter strains
P4	P1042
ST180	P980
TIGR4	P1104

During this experiment, the stabbed strain TIGR4 can secrete bacteriocin that interacts with the histidine kinase of the overlay strain P1104 as seen through the breakdown of X-gal as shown in Figure 3.4A. X-gal is a chromogenic substrate for β-galactosidase and bacteriocin interacts with it via histidine kinase where it marks site of enzymatic activity by forming dark blue precipitate. This indicates that the integrated *lacZ* promoter is functional and hence indicates an active *blp* locus. However, all other stabbed strains including TIGR4 KO strain did not produce bacteriocin that activates transcription of the *blp* locus in the reporter strain, or they

may not secrete bacteriocin, and thus the breakdown of X-gal did not occur (3.4B), except TIGR4 strain which produces bacteriocin against *S. parasanguinis*.

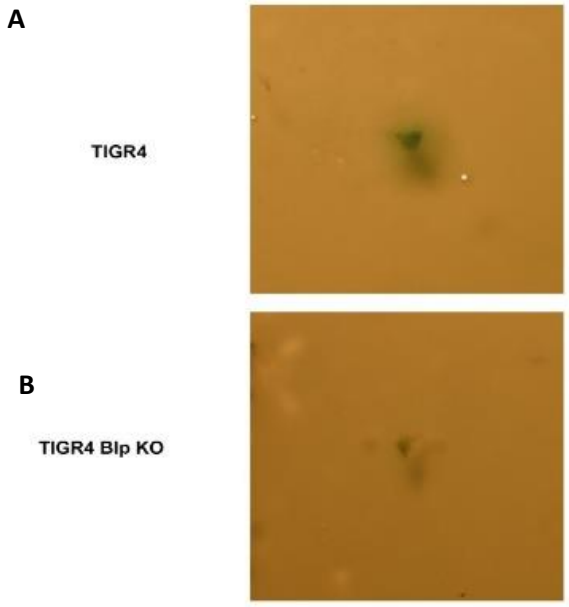

Figure 3-4: The stabbing of strains TIGR4 and TIGR4 KO into TSA plate and incubation. (A) Strain TIGR4, a BlpC secretor and contain an integrated lacZ gene, was stabbed into a TSA plate and incubated for 6 hours. Strain P1104 reporter with a deletion in BlpC, was inoculated into the overlay mixture with the addition of x-gal. Photos were taken after overnight incubation. The presence of an active galactosidase enzyme encoded by the lacZ gene resulted in the breakdown of x-gal to produce an intense blue colour. (B) strain TIGR4 KO, with a deletion in the blp locus, was stabbed into a TSA plate and incubated for 6 hours. Strain P1104 reporter with a deletion in BlpC, was inoculated into the overlay mixture with the addition of x-gal. Photos were taken after overnight incubation. The absence of active blp locus results in inactivation of glactosidase encoded by the lacZ gene did not breakdown the x-gal to produce an intense blue colour.

The reporter strain P1104 responded to the TIGR4 BlpC peptide by producing of beta-galactosidase, which can be visualised by the cleavage of X-gal to give a blue colour while the TIGR4 Blp knockout (KO) did not secrete BlpC (Figure 3.4). We then assayed different *S. pneumoniae* strains for bacteriocin production using the overlay assay. The producer strain included TIGR4 (wild type) and TIGR4 Blp knockout. TIGR4 (wild type) showed activity against both *S. S. parasanguinis, S. salivarius* and TIGR4 knockout. The TIGR4 knockout with a mutation in *blp* locus was bacteriocin-negative. The results of overlay assays are summarised in Table 3.3. As shown in Figure 3.5, the producer strain TIGR4 WT secreted bacteriocin which

killed *TIGR4 blpKO* and *S. parasanguinis*. On the other hand, TIGR4 blpKO producer strain did not secrete bacteriocin, hence no inhibition.

Table 3.3: Results of overlay assays.

Reporter Strains	Producer strains			
	TIGR4	**ST180**	**P4**	**D39**
1104	_	_	+	NA
1042	_	_	+	_
940	_	+	_	NA
S. parasanguinis	+	_	_	_
S. oralis	_	_	_	_
S. sanguis	_	_	_	_
S. mutans	_	_	_	_
S. mitis	_	_	_	_
TIGR4	NA	NA	NA	+
D39	+	NA	NA	NA
S. salivarius	+	NA	NA	NA
Streptococcus anginosus	-	NA	NA	NA
Streptococcus equinus	-	NA	NA	NA
Streptococcus faecalis	-	NA	NA	NA

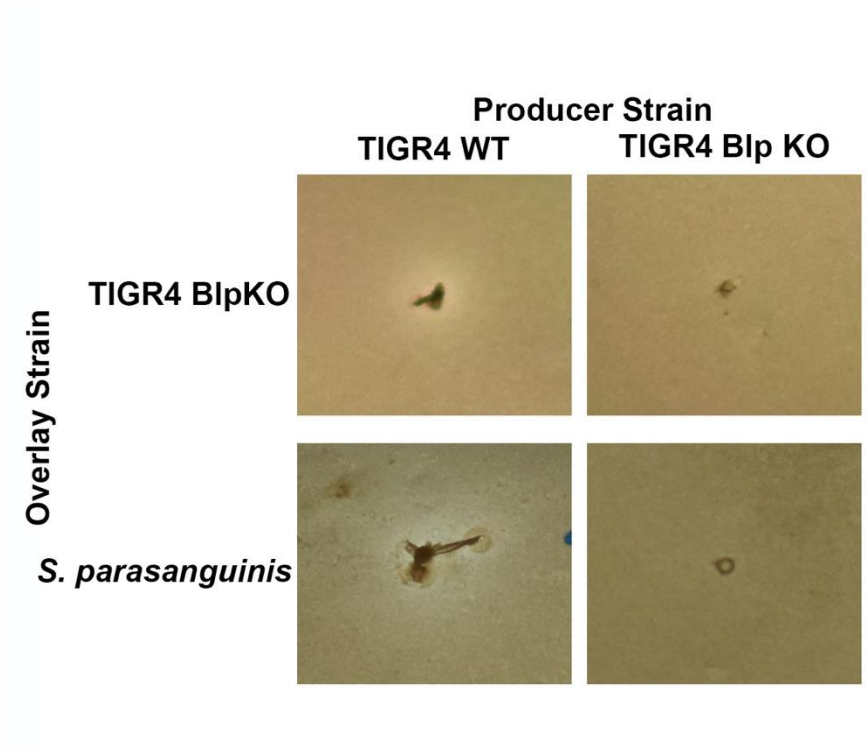

Figure 3-5: Bacteriocin-like activity in *S. pneumoniae*. The producer strain TIGR4 WT secreted bacteriocin which killed TIGR4 blpKO and *S. parasanguinis* evidenced by the appearance of zone of inhibition around the spiked producer (positive). TIGR4 blpKO producer strain did not secrete bacteriocin hence no presence of zone of inhibition (negative).

3.3.2 The effect of pH (slight acidity-pH 6 versus slight alkalinity pH 8)

Studies show that while the promoter driving expression of the two-component regulatory system *blpR/H* is constitutive, the remaining *blp*-promoters that control bacteriocin expression, immunity, and the inducer peptide BlpC, are pH-dependent and induced in the late exponential phase (Kjos et al., 2016). The competence system is highly sensitive to environmental cues including pH and exposure to certain antibiotics (Kjos et al., 2016). To determine the role of different pH levels and antibiotic stress, overlay assays were conducted and assayed for bacterial inhibition to the TIGR4 secreted bacteriocin. The effects of slight acidity (pH 6) and slight alkalinity (pH 8) on the differences in TIGR4 activity compared to the control producer strain P4 were compared. As shown in Figure 3.6, the producer strain P4 secreted bacteriocin against P1104 at pH 8 but not at pH 6 while the producer TIGR4 produced bacteriocin against *S. parasanguinis* at both pH6 and pH8.

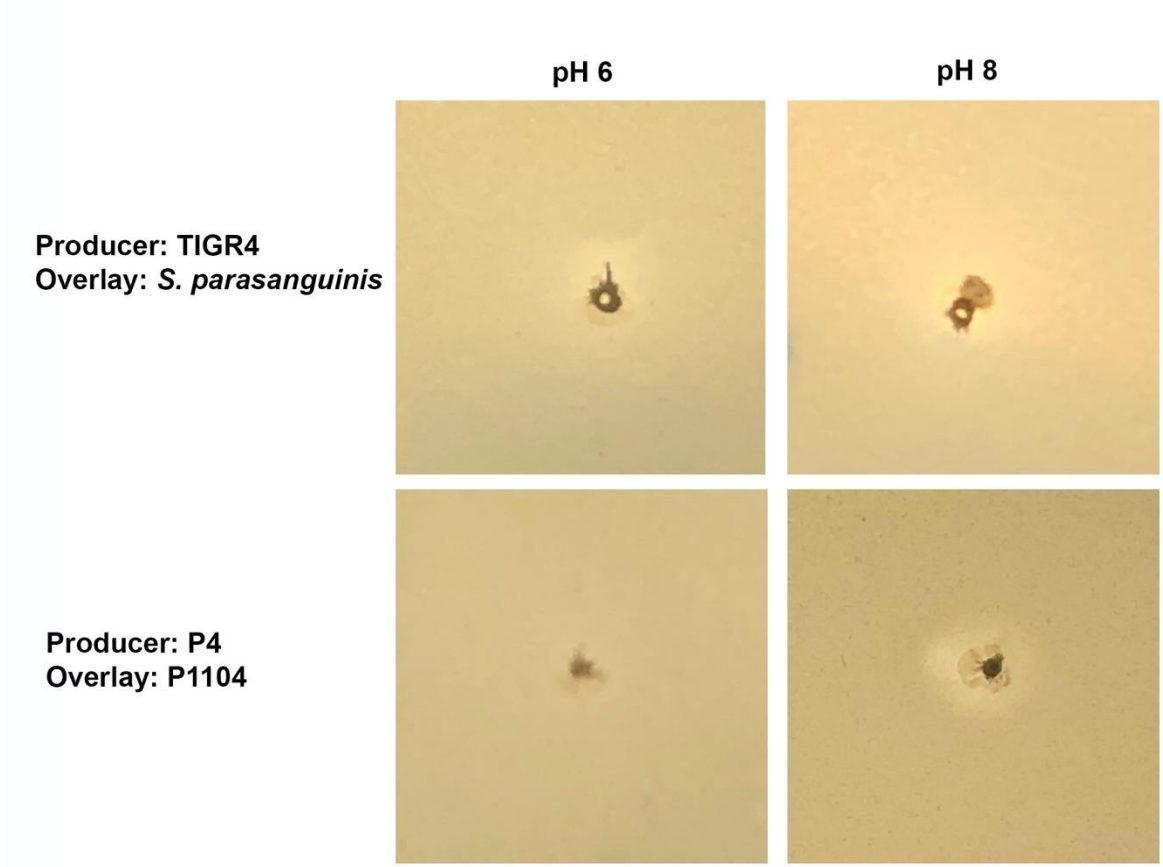

Figure 3-6: Overlay assay of TIGR4 and P4 as the producer strain and S. *S. parasanguinis* and P1104 as the reporter strain at pH 6 and pH 8 The producer strain P4 secreted bacteriocin against P1104 at pH 8 (indicated by clear zone of inhibition/clearance) but not at pH 6 (No zone of inhibition) while the producer TIGR4 produced bacteriocin against *S. parasanguinis* at both pH6 and pH8 (indicated by clear zone of inhibition/clearance).

3.3.3 Inhibition assay

A various number of techniques have been used to determine the activity of bacteriocins. We have developed a novel test based on co-culture inhibition. The assay is based on co-culture of the producer strain with the reporter strain and incubated for 6 hours with growth measured at hourly intervals in a ratio of 1:1 in a 15-ml tube; Agar plates supplemented with the desired antibiotic was used to culture the reporter strains. To screen for bacteriocin activity, serial dilutions were prepared, and the plates were incubated overnight in 5% CO_2 to allow sufficient time for the reporter strain to grow. The numbers of colonies were recorded, and results were plotted using Prism software. The performed inhibition assay shows rapid cell death: a 3-log

change within 90 minutes with TIGR4 against TIGR4 knockout (Figure 3.8). Since CSP stimulates competence when it reaches a critical concentration in the extracellular medium (in liquid culture of *S. pneumoniae* (Shanker and Federle, 2019), it was important to test the effect of CSP on the growth of TIGR4. Initially, the addition of CSP decreased colonies of TIGR4 only in the first 50 min of culture, beyond which it had no effect (Figure 3.7).

On the other hand, inhibition assay with direct co-culture of producer, TIGR4, and target, *S. parasanguinis*, selected by resistance to optochin, results in rapid cell death but recovery after 240 minutes (Figure 3.8). These could also mean that *S. parasanguinis* developed resistance against TIGR4 bacteriocin after 240 minutes. However, this hypothesis will be tested in the following chapters.

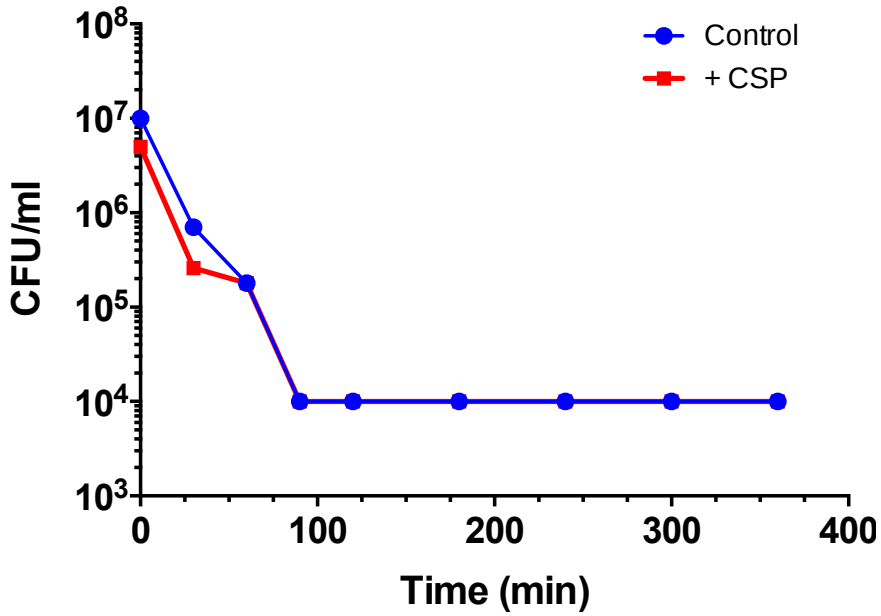

Figure 3-7: Representative growth curves of TIGR4 in cultures treated with CSP or without (control). Initially, the addition of CSP decreased colonies of TIGR4 only in the first 50 min of culture, beyond which it had no effect. Points are the means of 3 reads; errors were less than 5%. The experiment was repeated on 3 further occasions with similar results. Two-way ANOVA statistical testing showed significant P value=<0.0001.

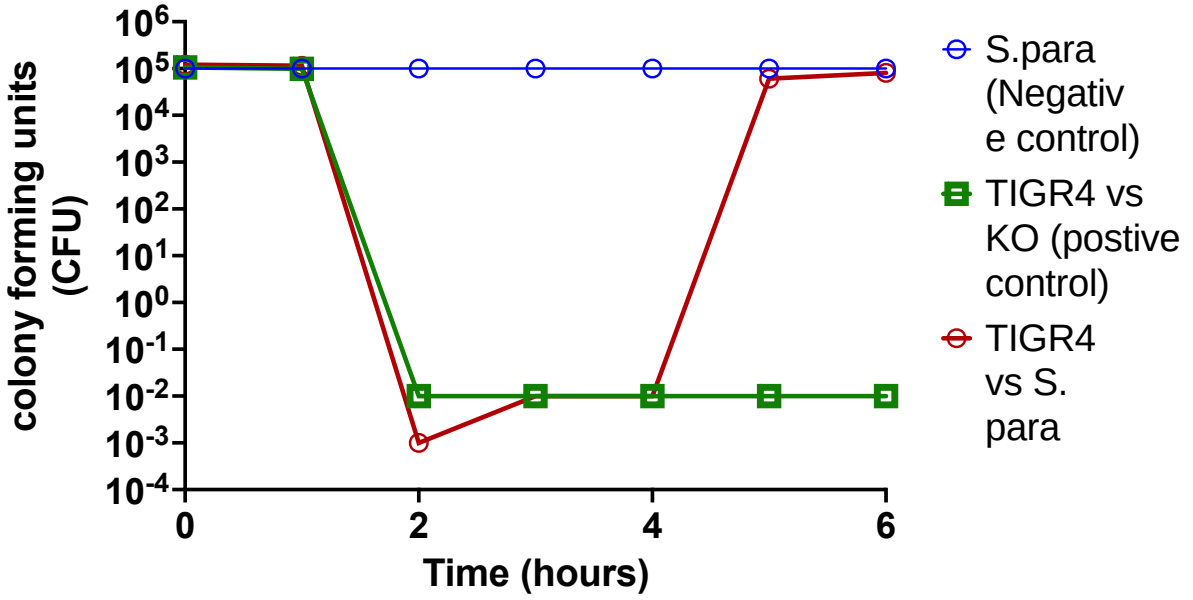

Figure 3-8: Representative growth curves of S. parasanguinis (Negative control), TIGR4 vs KO (positive control) and TIGR4 vs S. parasanguinis at different time points.The graph shows rapid cell death but recovery after 240 minutes for TIGR4 vs S. parasanguinis, rapid death without recovery for TIGR4 vs KO (positive control) and no effect on S. parasanguinis (Negative control). Points are the means of 3 reads; errors were less than 5%. The experiment was repeated on 2 further occasions with similar results. Two-way ANOVA statistical testing showed significant P value=<0.0001.

3.3.4 DNA Sequencing

The DNA extracts were sequenced in 2 x 300 bp paired-end using Illumina sequencing technology, sequencing by synthesis (SBS) on a MiSeq platform at the Glasgow Polyomics facility. The Illumina sequencing technology was chosen because of its versatility and is widely adopted next-generation sequencing (NGS) technology, accounting for at least 90% of the world's sequencing data (Illumina Inc., 2021). Illumina sequencing technology is a popular method for DNA sequencing because it is high-throughput, accurate, and relatively low-cost. The MiSeq platform is a benchtop sequencer that uses the Illumina technology and it can produce both short and long reads, enabling the user to get a high coverage of the genome. Sequencing by synthesis (SBS) is a method of DNA sequencing that uses reversible terminator nucleotides, which are incorporated into a growing strand of DNA during synthesis. These

nucleotides have a fluorescent label that is read by a detector as the incorporation occurs, providing real-time information about the sequence. This method is sensitive and enables accurate base calling. The Glasgow Polyomics facility was chosen to use the MiSeq platform with SBS technology because it offers the desired level of accuracy and throughput for their sequencing needs, and it is appropriate for the specific samples or experiments they are working on. Additionally, MiSeq is a benchtop sequencer which means it can be operated with less specialized technicians, and it is cost-efficient.

Our DNA samples were bar-coded and ran simultaneously together. This was amenable in the Illumina sequencing instruments and reagents, which immensely support parallel DNA sequencing. The Illumina sequencing by SBS technology supports both single- and paired-end library reading. This technology also has capabilities of short-insert paired-end reads for high-resolution genome sequencing and long-insert paired-end reads for efficient sequence assembly and *de novo* sequencing. The combination of short and long inserts provides the advantage of longer reads increases the ability to fully characterize any genome (Illumina Inc., 2021).

In the present study, the short-read sequences were processed by Glasgow Polyomics using a program called Trimmomatic to remove very short reads (< 36 bp), adaptor sequences, trailing low-quality reads at the 5’ and 3’ end of the reads, and any sequences with low quality. Trimmomatic was chosen because it allows for more flexibility in trimming Illumina sequence data (Bolger *et al.*, 2014). According to Bolger et al. (2014), despite the many available NGS read pre-processing tools, no single or a combination of the pre-processing tools offers versatility and accurate handling of paired-end data and high performance. These limitations led to the development of the Trimmomatic to overcome them (Bolger et al., 2014).

3.4 Discussion

The present study has demonstrated that *S. pneumoniae* TIGR4 strain produces bacteriocin that facilitates the inhibition of TIGR4 blpKO, *S. salivarius* and *S. parasanguinis* rival strains. TIGR4 bacteriocin production was not affected by pH, unlike the P4 producer strain. Furthermore, CSP initially suppressed the growth of TIGR4 (in the initial 50 minutes). A co-culture of TIGR4 WT and *S. parasanguinis* revealed the growth of *S. parasanguinis* was briefly suppressed by TIGR4. These could also mean that *S. parasanguinis* developed resistance against TIGR4 bacteriocin after 240 minutes or reduced growth of TIGR4 due to bacterial cell lysis, and hence reduced or no bacteriocin produced. However, this warrants further investigation in the preceding chapters. The outcomes were moderated by *S. parasanguinis* (Negative control) and TIGR4 vs KO (positive control). As expected, there was no change for the *S. parasanguinis* grown alone (Negative control) while the TIGR4 vs KO (positive control) had a standard deviation of 11532.56259 and 7094.598885 for times 0 and 1 respectively. This shows that production of bacteriocin that facilitates the inhibition of both TIGR4 blpKO and *S. parasanguinis* rival strains, was effective for the first 240 minutes before reducing.

"KO" strain typically refers to a "knockout" strain, which is a bacterial strain that has had a specific gene inactivated (or "knocked out") through genetic engineering. Comparing the bacteriocin production of TIGR4 to that of a KO strain can be used to help understand the role of specific genes or pathways in the production of these antimicrobial peptides. In this case, we knocked out genes known to be involved in bacteriocin production in TIGR4 strain to compare the bacteriocin production of the KO strain to that of the wild-type strain (TIGR4) to see if the knocked-out gene is indeed involved in bacteriocin production. The results as indicated in the chart above is affirmative.

The presence of *blp* locus in pneumococci has been known for several years, however, there is only a little information regarding the functional characteristics of the various proteins encoded

by the *blp* locus. We could identify bacteriocin-like activity of few strain members of *S. pneumoniae* using *in vitro* assays for either of the three strains for which the locus has been best characterized, (TIGR4, P4, and D39). While the present study has demonstrated that TIGR4 does have an active blp locus, we have interestingly found that TIGR4 can inhibit the growth of *S. parasanguinis in vitro* assays. The inhibition ability of TIGR4 can be attributed to *blp* gene. The requirement of the *blp* gene suggests that *blp* locus encodes a two-peptide bacteriocin, pneumocin MN (Eijsink *et al.*, 2002; Nissen-Meyer et al., 2009). Genes involved in signal transduction through two-component systems have been identified as virulence factors in disease models in various studies, but no published studies have included IE. TCSs are common in bacteria and are composed of a membrane-bound protein called a histidine kinase (BlpH) and a corresponding cytosolic response regulator (BlpR) protein. The BlpH protein senses environmental changes, autophosphorylates a histidine residue, and signals to its corresponding cytosolic BlpR protein by transferring the phosphate to an aspartate residue. The BlpR undergoes a conformational change and induces a response by regulating gene transcription (Trihn M, et al, 2013). Deletion of the entire *blp* locus resulted in a loss of both bacteriocin production and immunity, suggesting that proteins involved in bacteriocin production and immunity to the Blp bacteriocins is encoded in this operon. This was demonstrated previously by de Saizieu et al. (2000), where a mutant strain with single deletion of the entire *blp* locus was deficient in bacteriocin production and immunity (de Saizieu et al., 2000). The gene encoding for bacteriocin production and immunity is likely the open reading frames (ORFs) encoding the bacteriocin-like peptides identified within *blp locus.* A previous study using microarrays analysis has shown that the application of purified BlpC results in the upregulation of genes within the *blp* locus only (de Saizieu et al., 2000). We have attempted to synthesize or purify the BlpC protein but we could not, most likely, because of the BlpC highly hydrophobic nature. The loss of intra-species inhibition with mutations in *blp* locus shows that

the *blp* locus makes a significant contribution to bacteriocin production. Because the TIGR4 strain can inhibit the growth of *S. parasanguinis*, either the entire *blp* locus in *S. parasanguinis* is inactive or missing, including the production of immunity proteins, or the activity of the bacteriocin produced by TIGR4 are sufficient to result in a failure of the *S. parasanguinis* immunity protein to protect against the TIGR4 bacteriocins produced. *S. pneumoniae* has persisted as an inhabitant member of the nasopharynx and a cause of significant disease heavily because of its remarkable ability for adaptation through horizontal gene transfer. Competence allows pneumococci to fix chromosomal damage through the uptake of homologous DNA from the surrounding environment (de Saizieu et al., 2000; Stevens *et al.*, 2011). Studies of many bacterial populations have demonstrated that most DNA that is incorporated into the pneumococcal genome is derived from other pneumococci (Chewapreecha et al., 2014; Croucher et al., 2011). The direct source of DNA for these recombination events is more likely to come from the neighbouring bacterial cells. Studies of pneumococci have shown that most pneumococci secrete one of two major CSP types and have an intact competence system. In nature, presumably, neighbouring pneumococcal strains that share the same CSP type are more likely to communicate through the same induction of the competence state, which results in the production of bacteriocins and immunity proteins.

Unlike the competence system, the *blp* bacteriocin locus is characterized by diversity in the signalling peptides pheromone as well as the immunity proteins (de Saizieu et al., 2000; Lux et al., 2007; Reichmann and Hakenbeck, 2000; Son et al., 2011). Pneumococcal strains can produce one of four major BlpC types and may produce a variety of different pheromones with their associated immunity proteins. Given this diversity in signalling and produced proteins, competence-mediated induction of the *blp* locus could provide a competitive advantage to pneumococcal strains beyond that provided by bacteriocin production alone. bacteriocin production by itself may be sufficient to provide DNA from a wide variety of neighbouring

pneumococcal strains but cross-regulation of competence and bacteriocin production may enhance the exchange of DNA by coordinating the timing of the two systems. Because we have studied the role of *blp* locus and competence effect on bacteriocin and immunity proteins production in pneumococcal strain, these systems are required for bacteriocins and immunity proteins production.

We have demonstrated that strains carrying a deletion in the *blp* locus do not secrete BlpC pheromone and have no inhibitory activity in both overlay and inhibition assays. In this study, we showed that Blp bacteriocins can eradicate closely related pneumococcal strains. The contribution of bacteriocins to inhibition ability is most notable in strains that lack a functional *blp* locus, such as *S. parasanguinis*, *S. salivarius* and TIGR4 KO strains. The role of *blp* locus will be particularly important in populations where competence is likely to be induced. This data suggests that a competence system may enhance the production of bacteriocin after CSP stimulation. Unlike TIGR4 wild-type strains, we suspect that the TIGR4 KO strain may not produce bacteriocins or immunity proteins due to the absence of *blp* locus. We found that *blp* locus deletion strains, TIGR4 KO, that do not express bacteriocin and immunity proteins showed dysfunctional bacteriocin induction even in the presence of CSP.

Our studies have shown that the bacteriocin mediates the production of bacteriocins and immunity proteins. This implies that cheater strains that were previously described to only produce immunity proteins but not bacteriocins in response to BlpC production by competitors may retain that ability to secrete their BlpC during the competence state. Due to competence to be considered a stress response, the mechanism may become crucial during the bacteriocin-mediated inhibition, potentially allowing a strategy of self-protection. This hypothesis was not tested in our study because we have not tested cheater strains with disrupted BlpC gene although it does encode immunity proteins that would protect against bacteriocin-producing strains. delete the BlpC gene

Our studies did not address whether bacteriocin secretion itself may also be supported by the BlpC gene. In the genetic modification attempt, deletion of the BlpC locus using inverse PCR with primers was not successful. This because transformation insert resulted in cytotoxic effects.

Overlay assay is a rapid technique to determine the range of activity for bacteriocin production by *S. pneumoniae,* and to demonstrate competitive interactions that may occur among different pneumococcal strains. Overlay assays could be adapted to be used for screening multiple strains for bacteriocin production on a single agar plate. We have successfully screened different pneumococcal strains with this assay by using both stab and well methods to screen bacteriocin production.

S. pneumoniae antimicrobial peptides that are regulated in a density-dependent manner, are well suited to detection by overlay assays, as this allows growth to a higher density which may not achieved when organisms are grown in liquid culture. Growth on an agar solid medium might also enable reflecting the *in vivo* growth conditions of the nasopharyngeal tract.

Overlay assay has also some limitations. The overlay assay is qualitative. Hence, comparisons between the inhibitory activity of the pheromone secretion of pneumococcal strains must be made carefully. The growth rates and conditions should also be considered when comparing different strains because the outcome of the overlay assay relies greatly on the growth rate of the overlay and stabbed strains. Given the known diversity of genomic content in *S. pneumoniae* community, any inhibitory activity noted with overlay assay cannot be attributed straight away to the *blp* locus without removal of the locus responsible for pheromone production.

We describe here a modified assay for bacteriocin detection using bacteria grown in liquid culture. This worked very well and provides a more reproducible and quantitative assay of bacteriocin activity. It also revealed the growth of apparently resistant colonies of *S.*

parasanguinis that emerged sometime after co-culture, that would not necessarily be easily detected in an overlay assay.

To better understand the activity of the *blp* locus, independent of inhibitory activity, producer strains were tested using an overlay assay with a series of three previously described BlpC reporter strains. These reporter strains were constructed to contain one of three different *blpH* alleles that respond to the three most common BlpC pheromones that are secreted by *S. pneumoniae*. The indicator strains have an integrated *lacZ* gene that is controlled by a BlpH dependent promoter and carry a deletion in the *blpC* gene that prevents self-stimulation (Kochan and Dawid, 2013; Son et al., 2011). We hypothesised that producer strains that produce bacteriocins will activate the LacZ gene and break x gal to produce a blue colour in the plate overlay assay. This indicates that the integrated *lacZ* promoter is functional and hence indicates an active *blp* locus.

3.5 Conclusion

Genetic competence, bacteriocin, and immunity activity appear to be closely linked in Streptococci. The present study has demonstrated that TIGR4 does secrete bacteriocin, which can kill a number of targets. The capacity to produce bacteriocin under competitive conditions has proven to be a powerful advantage to eradicate organisms of the surrounding flora, underscoring the importance of considering the environmental inputs affecting bacteriocin production. The *Blp* locus is responsible for the production of both bacteriocin and immunity proteins in the TIGR4 strain.

CHAPTER 4: Cloning and Expression of a Putative Immunity Protein from TIGR4

4.1 Introduction to the Function and Mode of Action of Immunity Proteins of Class II bacteriocins

Class II bacteriocins are a specific class of antimicrobial peptides that function to protect bacteria from other bacteria of closely related species. These proteins are produced and secreted by bacteria and act by targeting the cell walls of other bacteria, ultimately leading to the death of the targeted cells. One specific example of a class II bacteriocin is the bacteriocin produced by TIGR4, which is produced by the bacterium *S. pneumoniae* (commonly known as pneumocin). The TIGR4 bacteriocin specifically targets the cell walls of other strains of pneumococcus and induces the process of lysis, which is the breaking open of the target cell walls. Understanding the mechanism of action of the TIGR4 bacteriocin and other class II bacteriocins is crucial for the development of new antibiotics. These peptides could potentially serve as alternatives to traditional antibiotics, which can have negative side effects on the human body and often lead to the emergence of antibiotic-resistant strains of bacteria.

4.2 Overview of TIGR4

TIGR4 variant strains of *S. pneumoniae* have been identified in areas where they differ from the reference 6A serotypes, and these areas are in their blpM and blpN peptides that induce inhibitory inhibition activity and can act as a 2-peptide bacteriocin when combined. In TIGR4 variants, the M and N peptides differ by 3 and 2 amino acids from those of the 6A serotypes, and that variability results in undetectable inhibitory inhibition capability when observed in overlay assays (Dawid et al., 2007). For this reason, the goal of this investigation is to determine if recombinant plasmids in susceptible bacterial hosts confer resistance to the TIGR4 wildtype produced bacteriocin through horizontal gene transfer and transform the entire TIGR4 blp locus.

While the usual recombinant method of transferring exogenous DNA non-sexually involves plasmids that are typically derived from the biotech work horse- *Escherichia coli* due to its ease of cultivation and relative safety when being used, in this particular transformation quest, *E. coli's* use as a host for transformation would be limited (Idalia and Franco, 2017). The limitation is as a result of *E. coli's* poor ability to efficiently translate and fold proteins successfully post-translation, as noted by Bryksin and Matsumura (2010), who used this as the basis for manufacturing broad host range plasmids which are used as knock-in/knock-out vectors for integrating large DNA sequences in the transformation of many species (Sabri *et al.*, 2013).

In this investigation, the expression vector used was the pBAV1K-T5-gfp which has a backbone of 2792 base pairs that facilitate other elements needed in protein synthesis and identification such as fluorescing proteins, cloning sites, and promoters. pBAV1K-T5-gfp expression vectors replicates efficiently in both gram-positive and gram-negative bacteria (Bryksin & Matsumura, 2010). The synthesis of the pBAV1K backbone was achieved using the elements of pWV01 ori-based plasmids and deleting the copy-number control mechanisms functional in the pWV01 (Bryksin and Matsumura, 2010). Such mechanisms in plasmids operate by limiting the supply of initiation factors or through inactivation of the initiator through dimerization and iteron-binding to promote replication (Das *et al.*, 2005; Nordström and Dasgupta, 2006). Iteron represent Rep protein-binding sites, which cleaves the plus DNA strand to initiate replication.

The resulting plasmid contains two inverted repeats IRI and IRII which are useful in converting single-stranded DNA to its double-stranded form, ORF C protein which functions to moderate the risk of runaway protein as well as T1 and t0 transcription terminators, which are inserted to prevent RNA read-through to the plasmid part. The expression vector is donned with a Kan^R selector, which are genes that confer resistance of the antibiotic kanamycin to vectors that

successfully take up the exogenous DNA while inhibition off the vectors that fail to transform (take up exogenous DNA) (Manna *et al.*, 2013). The vector also has a T5 promoter which is under the control of a lac operator and facilitates the initiation of transcription (Kawe *et al.*, 2009).

A study has shown that in *in vitro* assays for bacteriocin activity demonstrated that both the *blpM* and *blpN* genes are required for wild type intra-species inhibitory activity but not immunity (Dawid S *et al*, 2007). This study has shown that loss of *blpO* gene preserved wild type levels of activity in both inhibition and immunity. In addition, deletion of the entire *blpMNO* locus had a deficiency in both inhibition and immunity, suggesting that a gene in this locus contributes to the immunity phenotype. In this study, we aim to clone and express a putative immunity protein contained within the MNO locus that we have termed *blpMNO*, from TIGR4, and study its ability to confer immunity.

4.3 Why Plasmid and Cloning Is Required

The use of plasmids and cloning in the study of a putative immunity protein from TIGR4 allowed the researcher to manipulate and study the protein in a controlled and efficient manner. Plasmids are small, circular pieces of DNA that can be easily replicated and transported within bacteria, making them a useful tool in molecular biology research.

Cloning, the process of creating multiple copies of a DNA fragment, was used in combination with plasmids to study the expression and function of the gene of interest in a controlled environment. By cloning the gene encoding the putative immunity protein from TIGR4 into a plasmid, we can introduce the gene into a bacterial host and study its expression under different conditions. By using different cloning strategies, such as utilizing a host that is auxotrophic for a specific nutrient or an inducible system, we can have greater control over the experimental conditions and gain a deeper understanding of the protein's expression and function.

Additionally, plasmid cloning was used to ensure that the gene of interest is present in multiple copies in the host cell, increasing the chances of protein expression and making it easier for us to study the protein's properties and activity. This ultimately improves the efficiency of the study and allows for a more comprehensive understanding of the putative immunity protein from TIGR4.

The process of cloning and expressing a putative immunity protein from TIGR4 in *S. pneumoniae* requires a few steps. First, the plasmid choice is crucial as it will determine the type of vector used for cloning the gene of interest. In this case, the pBAV1K-T5-gfp plasmid will be used, which allows for high-level expression of the gene in *S. pneumoniae.* The next step is to extract the DNA from the TIGR4 bacteria and amplify the gene of interest using polymerase chain reaction (PCR). The amplified gene will then be purified and inserted into the pBAV1K-T5-gfp plasmid using restriction enzymes. The resulting plasmid will then be transformed into *S. pneumoniae,* which will then be grown in culture and selected for the presence of the plasmid.

To demonstrate the protection or lack of, from the immunity protein, various assays can be performed. For example, an assay to test the bacteria's sensitivity to different bacteriocins can be conducted to see if the expressed protein confers any bacteriocin resistance. Additionally, a Western blot or ELISA assay can be performed to confirm the expression of the immunity protein in the bacteria. Finally, the bacteria can be challenged with pathogens and the level of protection or susceptibility can be evaluated to demonstrate the ability of the expressed immunity protein to protect the bacteria from infection. Overall, the process of cloning and expressing a putative immunity protein from TIGR4 in *S. pneumoniae* using the pBAV1K-T5-gfp plasmid is a powerful tool to understand the function of the immunity protein and its potential use as a tool for protecting against bacterial infections.

4.4 Results

4.4.1 Modifications of the pBAVIK1-T5

The pBAV1K-T5-gfp plasmid possesses the gene encoding a green-fluorescent protein, which acts as a sensor system that verifies if the plasmid successfully took up the gene of interest and are synthesizing the desired protein genes. However, in this investigation, we wished to adapt this vector to allow expression of a pneumococcal protein with a His-tag to allow subsequent protein identification and possible purification. The vector was thus modified as follows. The GFP part was replaced with multiple cloning sites and a His-tag. The replacement was achieved through digestion of the gfp plasmid shown in Figure 4.1, with *Eco*R1 and *Pst*1 restriction endonuclease enzymes.

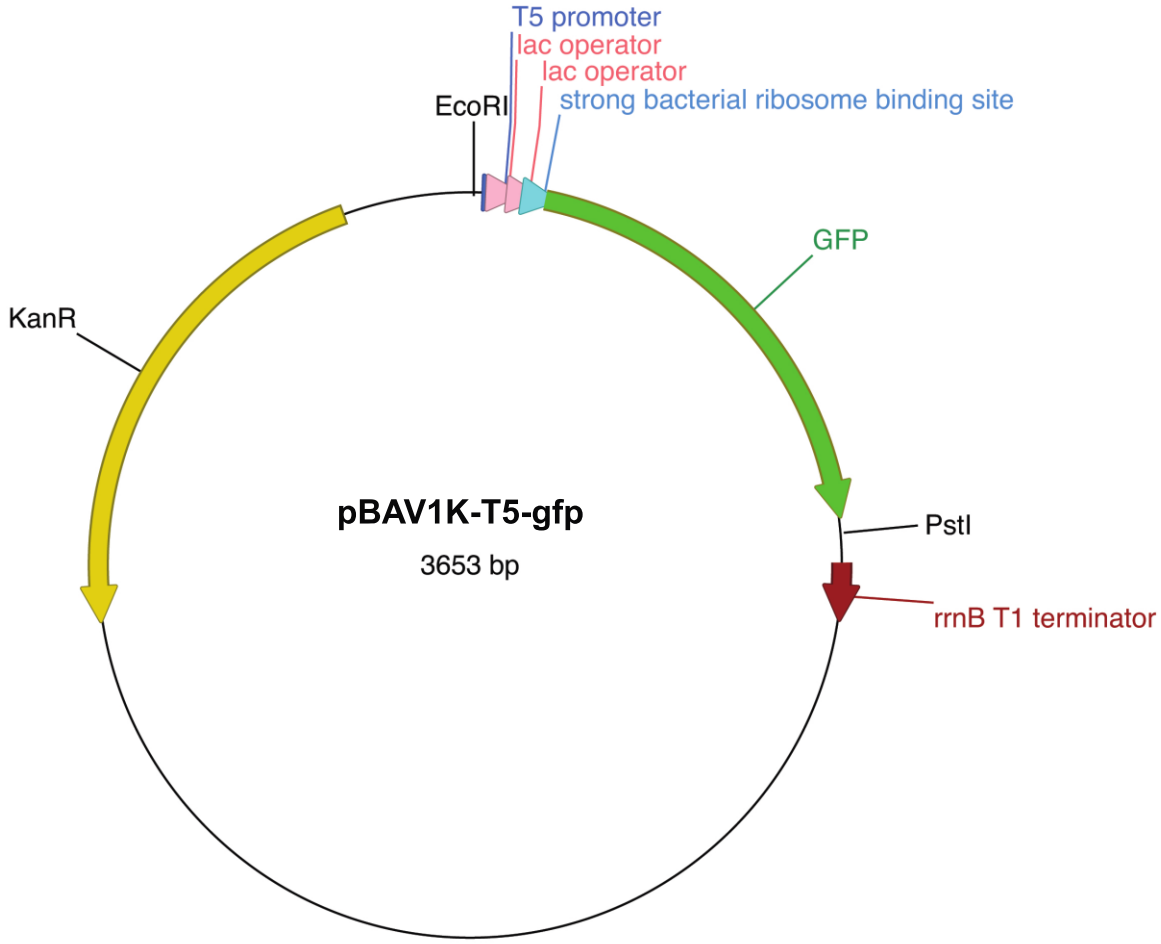

Figure 4-1: Shows the pBAV1K-T5 plasmid before digestion with EcoR1 and Pst1

The ribosomal binding sites, open reading frame, and the inverted repeats from the original vector, together with new multiple cloning site which included EcoR1-HindIII-Nhe1-Aro1-Pst1 (Figure 4.2), as well as the His-tag were then amplified using the primers below:

Forward:

GAAAGAGGAGAAATACTAGATGCACCATCACCATCACCATAAGCTTGCTAGC CTGCAGgcgc

Reverse:

ATGCGCCTGCAGGCTAGCAAGCTTATGGTGATGGTGATGGTGCATCTAGTATTTC TCCTCTTTC

Figure (2.4) shows PBAV-MNO-Map with restriction binding sites that have been used to construct the MNO immunity proteins.

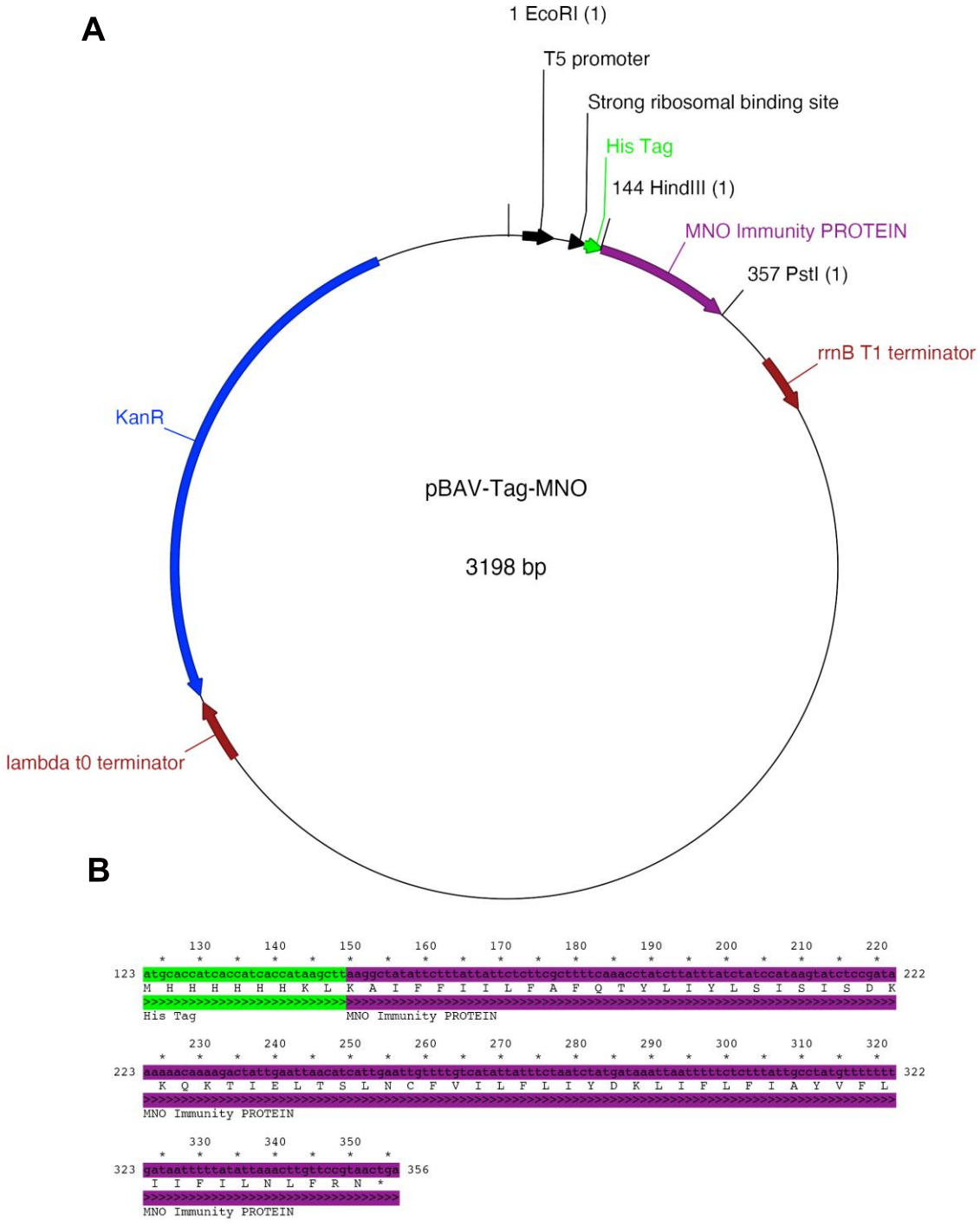

Figure 4-2 PBAV-MNO-Map with restriction binding sites. The indicated restriction binding sites have been used to construct the MNO immunity proteins.

The reverse primer provides an initiator codon (ATG) downstream of the ribosomal binding site, followed by an N -terminal His tag and a multiple cloning site (Figure 4.3)

Figure 4-3: A reverse primer with an initiator codon (ATG) downstream. Figure shows the complex of the multiple cloning site, promoter and His-tag downstream of the ribosomal binding site.

This complex was then digested using the restriction endonucleases (EcoR1 and Pst1) and the purified insert was placed in the EcoR1/Pst1 cut out vector, using EcoR1 and Pst1 binding sites. This then, created a new plasmid known as pBAV-Tag, which was transformed into *E.*

coli hosts and the transformed hosts selected by the Kan^R selection marker, as shown in Figure 4.3:

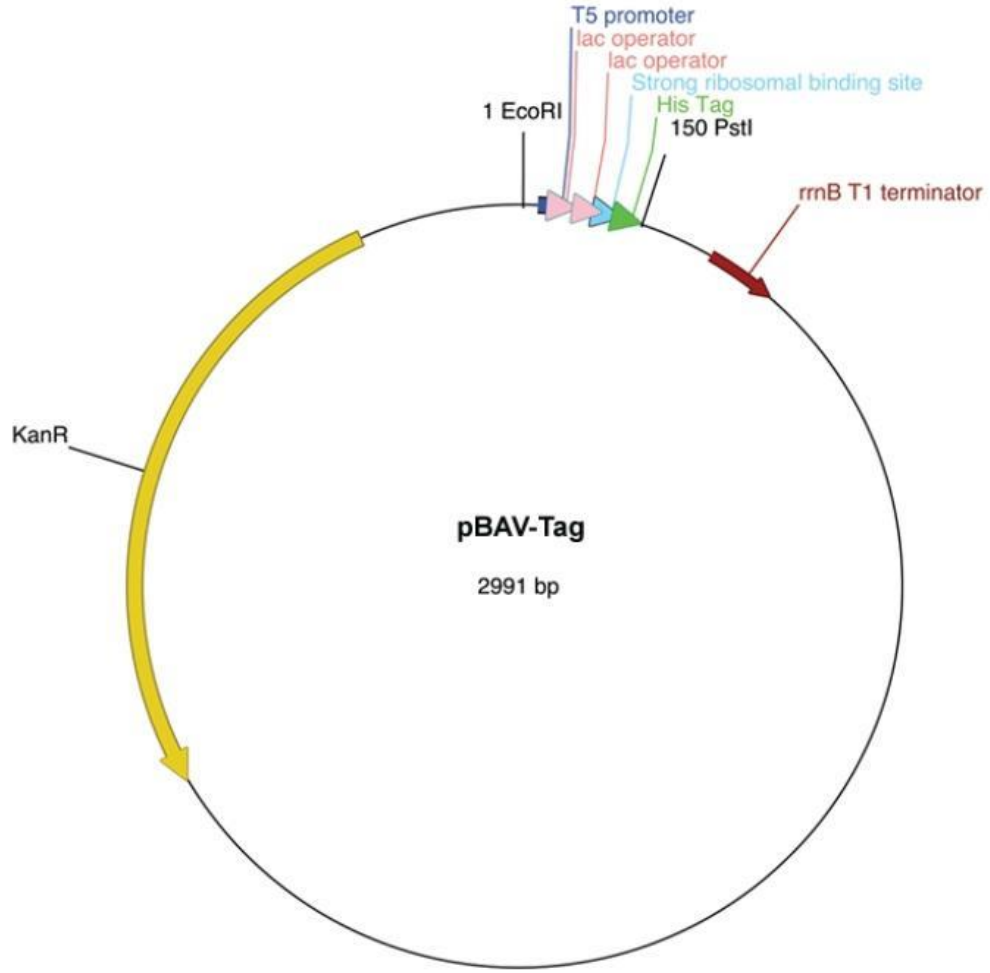

Figure 4-4: The structure of newly synthesized plasmid- pBAV1K-T5-gfp expression vector.

Overall, the plasmids were taken up successfully as confirmed through Kan^R selection where the hosts that successfully took up the vector also acquired a resistance to the antibiotic kanamycin, thereby forming colonies while the hosts that failed to take up the plasmid died once exposed to the antibiotic due to their lack of resistance. This then, confirmed a successful creation of the recombinant plasmid using the pBAV1K backbone.

4.4.2 BlpMNO amplification and uptake in pBAV-Tag

The basis of this investigation from the start has been to confer resistance to TIGR4 wild type strains of *S. pneumoniae* by using gene transfer techniques to insert modified immunity genes for blpMNO in the wildtype's blp locus. This particular locus in TIGR4 strains is known to have limited inhibitory inhibition activity, possibly due to dissimilar sequence structures in their blpM- and N bacteriocins. This makes it impossible for the strain to be potent enough to colonize the target area. Hence, putative immunity proteins in the TIGR4 genome were identified before being sequenced and amplified through PCR, using the primers containing a *Hin*dIII site upstream and a *Pst*1 site downstream. The primers used are shown below:

Forward: atgcgcAAGCTTaAGGCTATATTCTTTATTATT

Reverse: AtgcgcCTGCAGTTTGTATCCATATAGTGT

The product created was then cloned into the pBAV-Tag between using the HindIII and Pst1 sites as binding areas for the insertion, which produced pBAV-Tag-MNO. However, this recombinant plasmid was difficult to transform in *E. coli*, using the Kan^R selection method and thus, very few colonies were observed. There are a few explanations for this with the most probable one being rooted in the fact that Gram-positive and Gram-negative hosts interact differently with broad host range plasmids based on the structure of their origin Gram-positive hosts typically replicate via rolling circle (RC) and contain both single-stranded origin (SSO) and a double-stranded origin (DSO) (Jain and Srivastava, 2013). Single-stranded origins comprise single-stranded DNA and their recognition by the host's RNA polymerase as well as the synthesis of a short primer RNA are what are critical for the initiation of lagging strand synthesis (Jain and Srivastava, 2013).

Establishment of plasmids from Gram-positive bacteria in Gram-negative bacteria can be hindered by inefficient ssDNA conversion to ds-DNA forms. Secondly, a plasmid-encoded Rep protein- whose function is initiate RC-replication by making a site-specific nick at DSO,

that creates a primer for the leading strand, can fail to be expressed. Lack of expression of this protein is another contributing factor to broad host range plasmids typically designed for Gram-positive bacteria failing to transform in Gram-negative bacteria such as *E. coli* (Jain and Srivastava, 2013). If a plasmid has multiple origins that can be another factor to consider when transformation fails such as in this case, as the interaction of the host bacteria with the origins will differ significantly from the interaction in the next host (Jain and Srivastava, 2013). However, in this particular instance, because the *E. coli* had transformed successfully before it took up the immunity protein gene, these explanations were highly unlikely. It was discovered that the construct containing the BlpMNO gene had a mutation in which, based off isolates that were sequenced (Figure 4.7). Thus, this is suggesting that expression of this protein may be toxic to the cells.

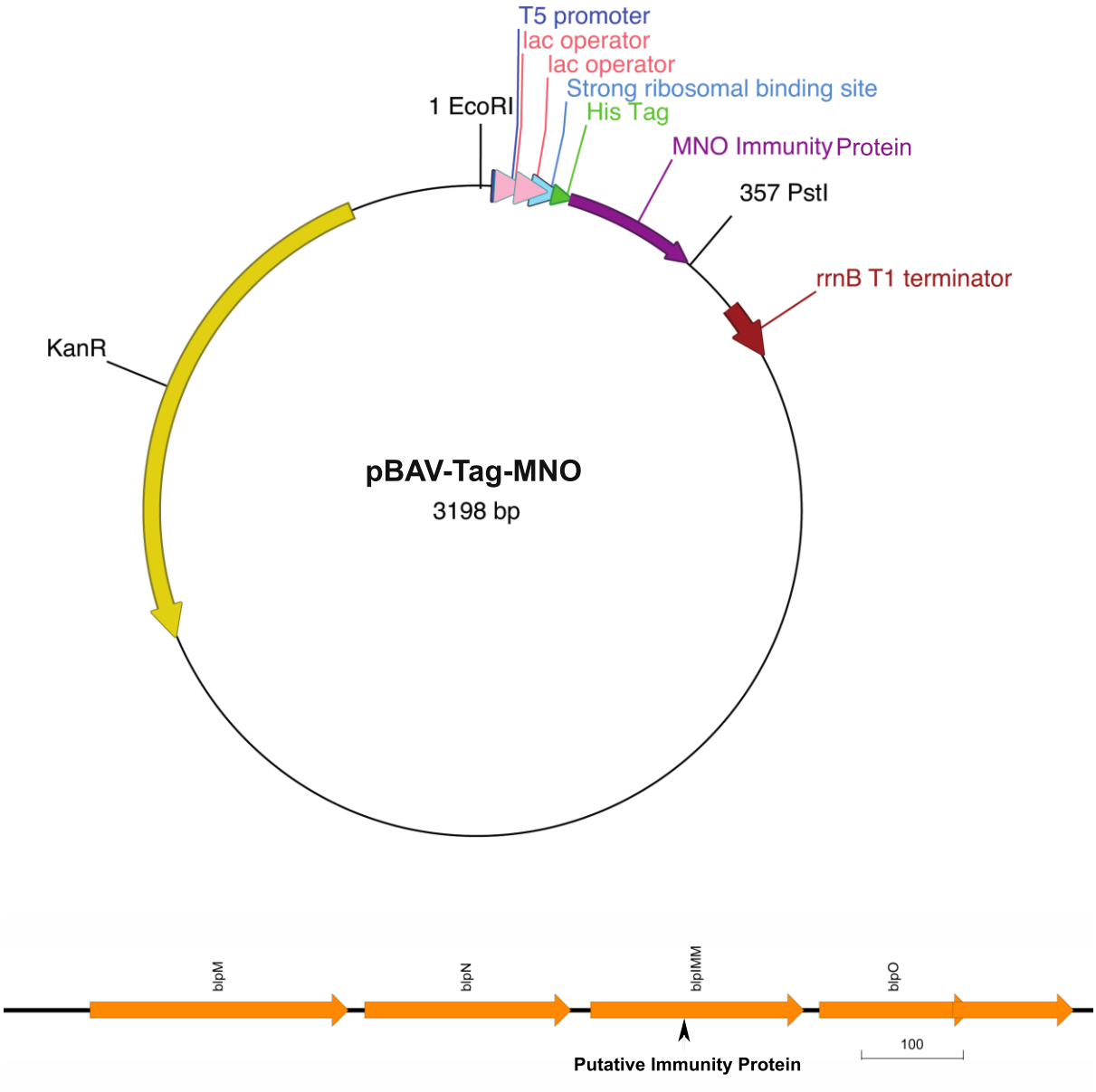

Figure 4-5: The diagram showing the final construct and the location of the putative immunity gene blpMNO in the blp locus.

The sequencing primers used are shown below:

Forward: CGGAGCCGATTTTGAAAC

Reverse: ATTAATTAAGCGGCGGCA

4.4.3 Rectifying the Immunity Protein Mutation Through Site Directed Mutagenesis

Once the mutation had been identified, rectification began with us resynthesizing the mutated fragment, together with its promoter, ribosomal binding sites as well as its EcoR1 and Pst1 cloning/restriction sites and producing the following sequence, with red highlights indicating points where the sites for EcoR1, HindIII (which is internally situated) and Pst1 are all located within the sequence shown in Figure 4.5:

atgcgcgGAATTCGCGGCCGCTTCTAGAGGAAATCATAAAAAATTTATTTGCTTTGTGAGCGGATAACAATTATA
ATAGATTCAATTGTGAGCGGATAACAATTACTAGAGAAAGAGGAGAAATACTAGATGCACCATCACCATCACC
ATAAGCTTAAGGCTATATTCTTTATTATTCTCTTCGCTTTTCAAACCTATCTTATTTATCTATCCATAAGTATCTCC
GATAAAAAACAAAAGACTATTGAATTAACATCATTGAATTGTTTTGTCATATTATTTCTAATCTATGATAAATTA
ATTTTTCTCTTTATTGCCTATGTTTTTTTGATAATTTTTATATTAAACTTGTTCCGTAACTGACTGCAGatgcgcg

Figure 4-6: Sequence of the mutated fragment. The sequence contains its promoter, ribosomal binding sites as well as its EcoR1 and Pst1 cloning/restriction sites.

Again, the synthesized fragment was cloned and inserted in the recombinant EcoR1/Pst1 cut vector. Also, this plasmid-fragment complex had an error which led to a premature stop. Thus, the Base-changer kit was employed to execute site-directed mutagenesis- a technique aimed at creating specific, targeted changes in double-stranded DNA (El-Gewely *et al.*; Kunkel, 1985). Through this method, the T nucleotide was introduced at the site where the deletion had occurred to restore the original sequence, similar to that obtained from the TIGR4 strain, which is shown in Figure 4.7 below:

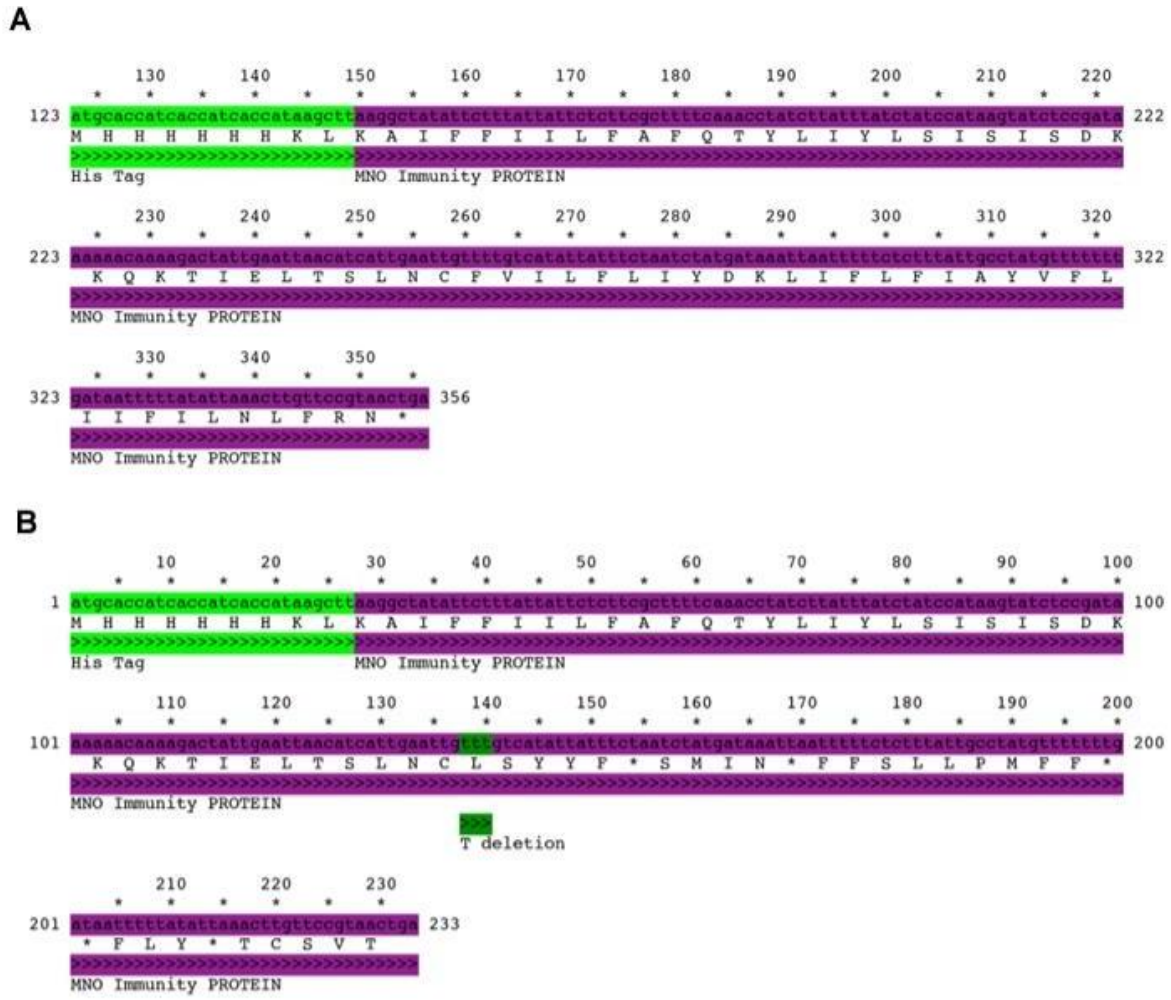

Figure 4-7: The rectified amino acid sequence of the MNO immunity protein. A) the corrected MNO immunity protein labelled with His-Tag with 4 T's. B) the MNO immunity protein labelled with His-Tag missing a T base leading to premature protein termination.

4.4.4 Transformation of the TIGR4 Wildtype and Other Streptococcus Strains

BlpMNO carrying plasmids that were replicated in *E. coli* were retrieved from the *E. coli* host and transferred to the TIGR KO as well as the *S. parasanguinis* strain, with the TIGR4 strain successfully transforming and taking up the modified immunity protein gene while *S. parasanguinis* failed to become transformed. Hence, there was no expression of the MNO immunity proteins. We aim to transfer it into TIGR4 wild type strain to assess if TIGR4 will be succussfully transformed with the DNAinsert. The insert was transformed successfully and Blp MNO was successfully expressed in TIGR4. We also tried to clone the BlpMNO gene in

TIGR4 KO. However, several attempts to remove the SJ cassette were unsuccessfull. This has to be included in future work.

To determine if the transformed *S. pneumoniae* expressed the MNO immunity protein, we extracted total proteins for the transformed strain and subjected them to Western blotting as described in the methods, using an antibody to the His-tag. There was no His-tag protein detected (data not shown).

We then assayed for the MNO immunity protein expression using immunofluorescence staining (Odell and Cook, 2013)with fluorescent antibodies directed towards the His tag as described in the methods section. The antibodies bind to the target species and fluorescence that is then visualized under a microscope (Figure 4.6).

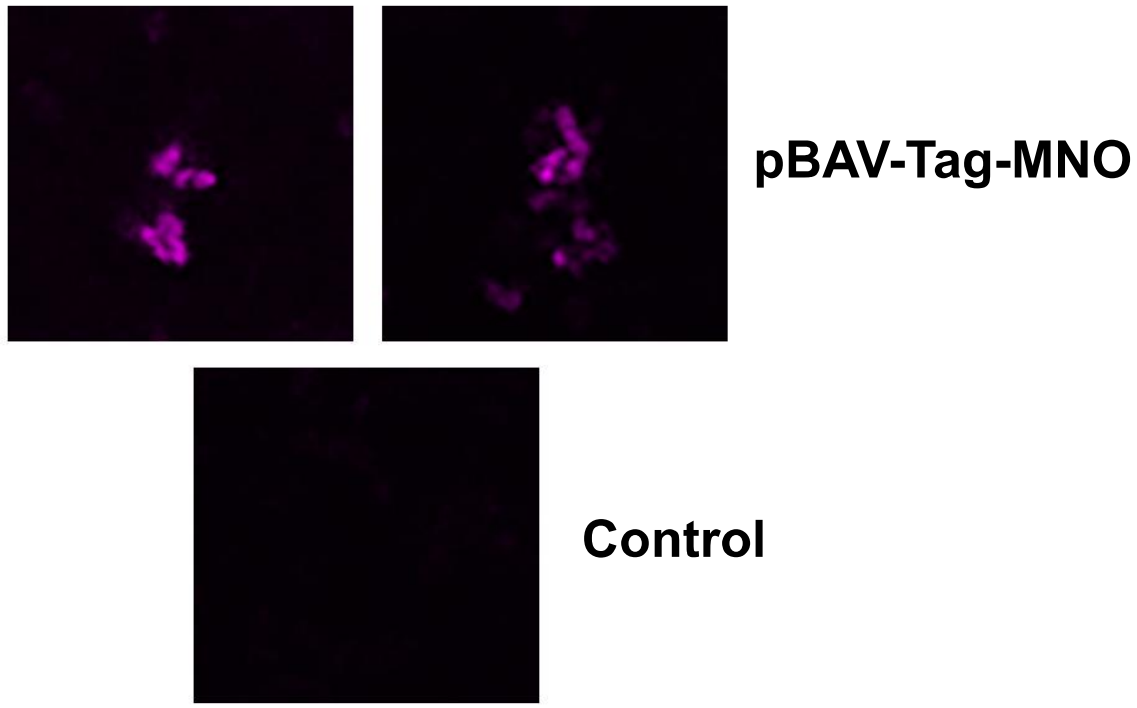

Figure 4-8: Assay for the MNO immunity protein expression using immunofluorescence staining. The upper two panels show TIGR4 cells transformed with MNO immunity protein labeled with His-Tag incubated with anti-his-tag antibodies. Expression of MNO immunity protein was visualised under immunofluorescent microscope. The lower panel (control) shows TIGR4 wild type with no expression of MNO immunity protein.

4.4.5 Site Specific Structural Predictions for N Terminal and the C terminals of Recombinant Protein.

Since site-directed mutagenesis methods are used to generate DNA sequences with mutated codons, insertions or deletions, allow the identification of the regulatory regions of genes while giving insight on the relationship between the protein structure and its function (Castorena-Torres et al. 2009: Eldelheit et al. 2016). Hence, the protocol used in most methods is based off modified steps of PCR, which it outlines, while showing the oligonucleotides used as primers and generating the regions on DNA where mutation codons need to be introduced to create a desired product. In this investigation, rectifying the deletion of T nucleotides at n=4 required an opposite action of inserting codons that related the amino acid gene structure of T in the sequence. The immunity protein is a transmembrane element with a helical structure and is common with transmembrane protein, they can assume different conformations to enhance its function as it interacts with membrane structures. The N-terminals lead the protein in its movement and conformation changes such that it is the first to exit as the protein moves as shown below in Figure 4.9:

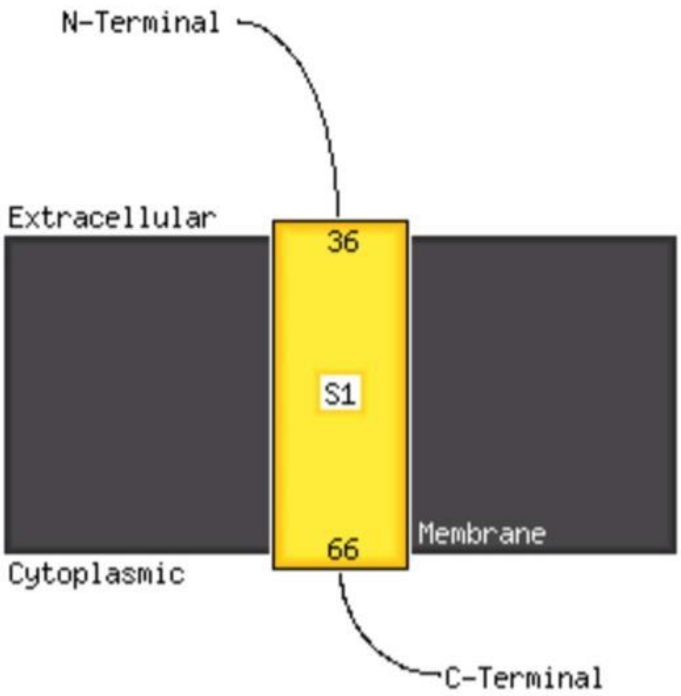

Figure 4-9: The predicted positions at N-(36) and C-(66) termini as the immunity protein operates.

The N-terminus also signals for precursor and signalling molecules that aide the protein in its quest and function. The C-terminal of a proteins is essential for binding, posttranslational modifications, and trafficking of the protein, as it contains mini-motif peptides and can play a role in transcription (Sharma and Schiller, 2019).

The terminals are identified by the number of residues they contain, with the N-terminal having the lowest residue number and the C-terminal having the highest residue number, as indicated in Figure 4.9. Binding of the protein to the membrane is achieved by the transmembrane domain as shown in Figures 4.8 and 4.9. This is the region of the immunity protein that spans the membrane using a hydrophobic domain that interacts with the hydrophobic membrane lipids.

4.5 Discussion

4.5.1 Function of bacteriocins in *S. pneumoniae* and Their Similarity to Antibiotics as Observed in This Investigation

In *S. pneumoniae*, this competitiveness is achieved through the synthesis of pneumocins that are synthesized on the blp locus. This gene locus encodes for bacteriocins and their cognate immunity proteins for various *S. pneumoniae* strains. However, variability within this locus results in some strains possessing inhibitory inhibition capacity while some strains lack it, making those that lack the capacity susceptible to those that possess it.

A key feature to all bacteriocin systems, is the co-production of immunity proteins along with active bacteriocins that act to prevent auto-destruction of the bacterium producing the bacteriocin. The mechanism by which putative immunity proteins confer resistance to pneumococcal bacteriocins is not known. Here we have begun to provide reagents that can address whether a putative immunity protein encoded in the BlpMNO locus can confer resistance to bacteriocin action. This will then allow further studies of its possible mechanism of action and the proteins with which it might associate. We have successfully cloned and expressed this immunity protein and it does appear to be a membrane protein from the immunofluorescence studies, as predicted form its sequence. How it confers immunity remains unclear; further experiments to express it in different TIGR4 constructs will be required in future work.

4.5.2 Plasmid Properties That Made It Ideal for Use in The Investigation

Studies such as transcriptional analysis, gene complementation or specific deletion of genes in the locus are vital in understanding the exact reason behind the toxicity of the section of the locus to *S. parasanguinis* and the TIGR4 KO strain but not TIGR4. However, one of the reasons for this is likely due to the presence of specific genes or elements within that section of the locus that are essential for the survival and growth of the TIGR4 strain, but are not present or

have different sequences in the other two strains. For example, the section of the locus may contain a gene encoding for a protein that is essential for the growth and survival of TIGR4, but not for *S. parasanguinis* or the TIGR4 KO strain. Additionally, this section of the locus may contain regulatory elements that activate certain genes in TIGR4, but not in the other two strains, leading to the toxic effect. It is also possible that the section of the locus contains elements that interact with other genes or pathways in the TIGR4 strain and this interaction is necessary for growth, whereas these interactions are not present in the other two strains, leading to the toxic effect.

The method of choice involved in conferring this resistance relied heavily on the uptake of exogenous DNA by bacterial plasmids before insertion in a host species. The plasmid of choice was genetically engineered from a plasmid whose origins were rooted in the cryptic plasmid-pWV01 ORI. Because it is a cryptic type of plasmid, its activity is undetectable in the host hence, its copy number needs to be controlled when it is in its native host (Bryksin and Matsumura 2010, p.1-2).

The resulting plasmid from this investigation had a pBAV-backbone that was attached to a polyhistidine tag (His-tag), multiple cloning site and a Kan^R selector. All these elements function well in a variety of hosts. Thus, the pBAV backbone becomes the site of connection for the cloning site, the His-tag and selectable marker. The His-tag was crucial in creating tagged proteins that could easily be purified by separation techniques such Immobilized Metal Affinity Chromatography (IMAC) and identified using anti His Tag antibodies. The multiple cloning site contained restriction sites that allowed the His-tag to be joined to the plasmid, as well as restriction sites where DNA could be inserted on to the plasmid as well as restriction sites that would allow the plasmid to insert the DNA, it was carrying into another cell. The Kan^R selector was to confirm if the plasmids had taken up the antibiotic gene that would allow them to be resistant to Kan resistance, and therefore show immunity to the bacteriocins of

surrounding strains that work in a similar fashion as antibiotics, to inhibit or kill competitor strains and species.

To prove its broad host-range nature, the plasmid was inserted in *E. coli*, which is a Gram-negative bacilli-shaped species that operates quite differently from Gram-positive bacteria such as *S. pneumoniae*, but can nonetheless, be transformed by plasmids of such species under the right conditions. In this way, the *E. coli* behaved as the positive control for this investigation. Its primary role was to show the functionality of the plasmid, before and after receiving the immunity protein gene. It also highlighted the areas of truncation in the recombinant protein and was able to be transformed by the vector once the protein was rectified. Meanwhile, *S. parasanguinis* became the negative control that received the modified immunity gene, just like the sample strain; however, it failed to be transformed by the exogenous DNA of the immunity protein.

This translated to no protein being synthesized when plasmids carrying the resistant-modified immunity genes were inserted in the cells of *S. parasanguinis*- a very different result from what was observed in the sample cells of TIGR4. The explanation for this is related to the bacteriocin-encoding genes, thus in spite of being in the same genus as *S. pneumoniae*, the bacteriocin and immunity nature of *S. parasanguinis* operates in a different manner from that of *S. pneumoniae* and is encoded for by genes such as Spaf 1859 and Spaf1860 (Geng et al. 2012, p.5).

4.6 Conclusion

The exact mechanism by which the putative immunity proteins confer resistance to pneumococcal bacteriocins is still not known. In this investigation, plasmids were used to study the function of the putative immunity protein. The plasmid of choice was a genetically engineered plasmid with a pBAV-backbone, a poly-histidine tag (His-tag), multiple cloning site and a Kan^R selector. The plasmid was inserted into E. coli, which served as a positive

control, to show the functionality of the plasmid before and after receiving the immunity protein gene. The investigation successfully cloned and expressed the immunity protein, which appears to be a membrane protein. However, further experiments are needed to understand how it confers immunity and the proteins with which it associates. While the nature of the TIGR4 immunity protein was successfully characterized and the protein modified, the process encountered a few setbacks along the way, which required rectification through more genomic analysis techniques. However, the end result did produce the desired outcome, which was successful expression of MNO protein in TIGR4. Nonetheless, the issue of the strain itself being unable to induce inhibitory effects on surrounding bacteria was not adequately addressed and should be addressed in future projects.

CHAPTER 5: Genetic Basis of Bacteriocin-Resistant *S. parasanguinis*

5.1 Introduction

S. pneumoniae serotype 4 (strain ATCC BAA-334 / TIGR4) is known for its Blp bacteriocin, which aids its survival competition for nutrition with other closely related strains and streptococci species. While Blp bacteriocin suppresses rival streptococci strains and species, there is an interesting observation of TIGR4 bacteriocin resistance in *S. parasanguinis*. This was observed in a co-culture of TIGR4 and *S. parasanguinis* strain in BHI media, where *S. parasanguinis* was able to grow in the presence of the TIGR4 produced Blp bacteriocin. We hypothesized that this unusual TIGR4 bacteriocin resistance is due to genetic mutations in the *S. parasanguinis* strain, which rendered it non-sensitive to the killer bacteriocin. Alternatively, like *Lactobacillus brevis* 174A (Noda *et al.*, 2018), it is also possible that the bacteriocin-producing TIGR4 strain has a self-resistance gene against self Blp bacteriocin, which could be transferable horizontally to *S. parasanguinis* when the two species are co-cultured. Mechanisms of horizontal gene transfer (HGT) include mainly conjugation and natural transformation. HGT by conjugation mechanism involves the transfer of DNA through a multistep process that involves cell-to-cell contact via cell surface pili or adhesins between donor and recipient bacteria. The conjugative machinery facilitates HGT encoded by genes on autonomously replicating plasmids or by integrative conjugative DNA elements in the chromosome (von Wintersdorff *et al.*, 2016). HGT by transformation involves the uptake of free fragments of extracellular DNA, their subsequent integration, and finally expressed functionally into the recipient cell. Other mechanisms of HGT include generalized transduction, where DNA loaded into the bacteriophage head is transferred to the recipient bacteria, and bacteriophage-like particles called gene transfer agents (GTAs) in cell lysates.

GTAs carry random DNA fragments when released through cell lysis from a donor cell's genome and spread to a recipient cell (von Wintersdorff et al., 2016).

Intra- and inter-species HGT by transformations between different strains of *S. pneumoniae* and closely related species was first reported in 1928 by Griffith (1928). This mechanism is commonly implicated in the transfer of antibiotic resistance genes. For example, by exposing penicillin- and streptomycin-sensitive *S. pneumoniae* to DNA from resistant strains, the strains acquired antibiotic resistance traits (Hotchkiss, 1951).

The present study was prompted by the eventual development of bacteriocin resistance in *S. parasanguinis* previously co-cultured with TIGR4 that produces Blp bacteriocin in BHI medium. Therefore, this study was guided by the hypotheses: (1) that bacteriocin resistance in *S. parasanguinis* is caused by mutations in bacteriocin-encoding genes; and/or (2) *S. parasanguinis* acquired self- bacteriocin-resistance genes from TIGR4 via horizontal gene transfer. Therefore, the present study aimed to test these hypotheses by genome sequencing analysis to ascertain whether there is a genetic basis for the Blp bacteriocin resistance in the resistant *S. parasanguinis*. Findings from this study are anticipated to be helpful in the identification of potential gene targets for TIGR4 bacteriocins and/or immunity mechanisms.

5.2 Results

5.2.1 Co-culture of TIGR4 strain with S. parasanguinis in BHI medium

To determine whether a TIGR4 rival strain, *S. parasanguinis,* is killed by TIGR4 bacteriocins, we co-cultured the two strains in the BHI medium. Figure 5.1 shows that the growth of *S. parasanguinis* was limited by TIGR4 bacteriocins at 1-2 hours, and remained suppressed up to 8 hours. After 3 hours, the growth of the *S. parasanguinis* reached about 10^5 CFUs when measured on an optochin agar plate. This data indicates that the *S. parasanguinis* is initially sensitive to TIGR4 bacteriocin, but the sensitivity was lost with time.

5.2.2 Confirmatory Resistance Test After Liquid Tube Assay

A confirmatory Blp bacteriocin-resistance test was performed on the bacteriocin resistant colonies of *S. parasanguinis*. Three Blp bacteriocin-resistance colonies of *S. parasanguinis* were picked from previous co-cultures of TIGR4 strain and *S. parasanguinis* in BHI medium and confirmed by the tube inhibition assay method. Briefly, in the liquid tube assay, bacteriocin-resistant colonies of *S. parasanguinis* were grown in tubes containing liquid BHI medium and re-tested for bacteriocin resistance in fresh direct co-cultures of the Blp bacteriocin producer TIGR4 WT strain and the target *S. parasanguinis*. Colonies of *S. parasanguinis* from the co-culture retained their resistance against TIGR4 bacteriocins as shown in Figure 5.1. This supports the conclusion that our target *S. parasanguinis* organism acquired a hereditary change that conferred its resistance. This validated our quest to proceed with the genome sequencing analysis to pinpoint whether mutations or HGT occurred in our target *S. parasanguinis* from the Blp bacteriocin producer TIGR4 WT strain. The Statistical findings are illustrated in the Table 5.1.

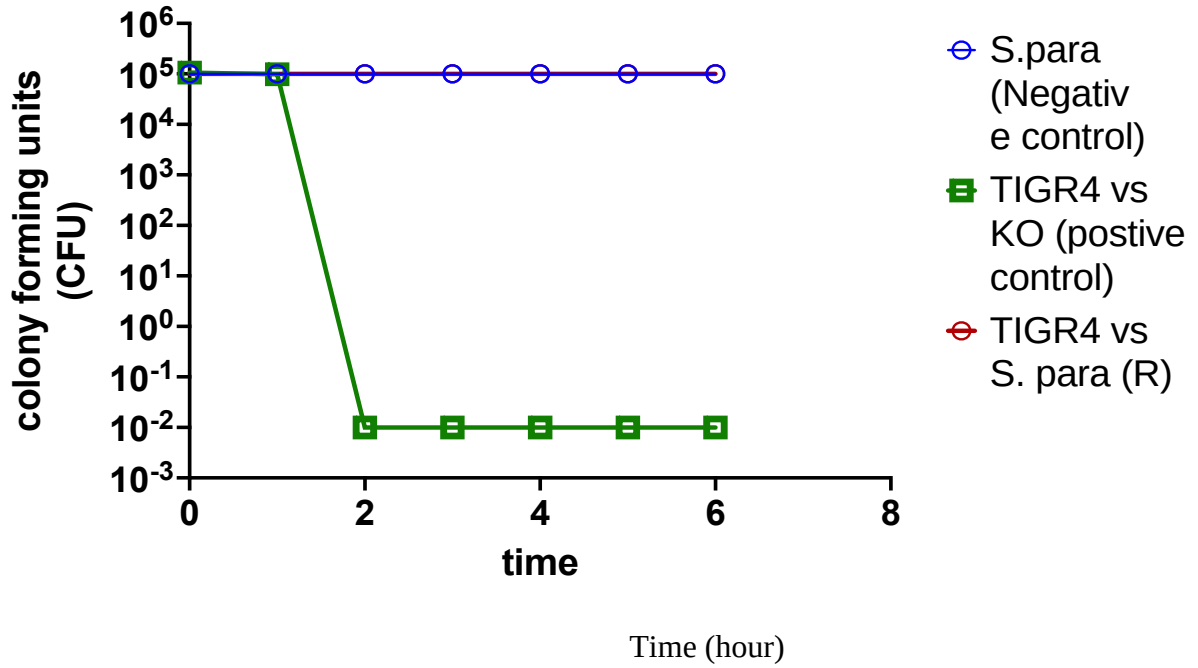

Figure 5-1: Inhibition assay on direct co-cultures of the Blp bacteriocin producer TIGR4 WT strain and the target S. parasanguinis cells in BHI media. Optochin resistance selected target cells revealed sensitivity in the first 2 h of co-culture, after which the resistance against TIGR4 bacteriocin increased and picked from 3-5 h. Values represent the mean CFUs (n = 3) of S. parasanguinis obtained from triplicate co-cultures plotted against time (h). Negative control is S. parasanguinis inhibition assay on direct co-cultures of the Blp bacteriocin producer TIGR4 WT strain and the resistant S. parasanguinis cells in BHI medium. Optochin resistance selected target cells revealed resistance against TIGR4 bacteriocin throughout the 6 h of the co-culture. Values represent the mean CFUs (n = 3) of S. parasanguinis obtained from triplicate co-cultures plotted against time (h). Two-way ANOVA statistical testing showed significant P value=<0.0001.

5.2.3 DNA Extraction

DNA was extracted from both Blp bacteriocin producer TIGR4 WT strain and the target bacteriocin-resistant *S. parasanguinis*. Briefly, three pure colonies of bacteriocin-resistant *S. parasanguinis* from the confirmatory co-cultures and one genetically uncontaminated WT *S. parasanguinis* as the reference strain from a separate pure culture were harvested and their respective DNA extracted separately and purified. Table 5.2 shows the concentration of the DNA yield extracted from the organisms. DNA yield from pure *S. parasanguinis* colonies from co-cultures with TIGR4 WT strain ranged from 70.6 to 162.3 ng/ml (mean 116.93±45.86 ng/ml). This was considerably lower than 143.70 ng/ml obtained from the reference strain. The purity of these DNA extracts was determined spectrophotometrically using NanoDrop®

Sample spectrophotometers at two wavelengths, 260 nm, and 280 nm. The ratio of absorbance readings at 260 nm and 280 nm were used to calculate DNA purity, where a ratio of ~1.8 was generally accepted as "pure" for DNA. As shown in Table 5.2, none of the extracted DNA products achieved the purity standard ~1.8. However, the DNA purity of the reference strain (WT) was 1.67, which was approaching the ~1.8 standard. The purity of the DNA extracted from the *S. parasanguinis* colonies from co-cultures with TIGR4 WT strain ranged from 1.4 to 1.67 (mean 1.50±0.10). While these DNA extracts can be considered appreciably impure and the impurity could be due to the presence of protein, phenol or other contaminants that absorb strongly at or near 280 nm.

Table 5.1: Yield and purity of DNA products extracted from pure colonies of *S. Parasanguinis* from co-cultures with TIGR4 WT strain and WT *S. parasanguinis* as the reference strain.

S. parasanguinis	**DNA concentration (ng/ml)**	**DNA purity @ 260/280 ratio**
Reference strain (wt)	143.70	1.67
S. parasanguinis (1)	117.90	1.50
S. parasanguinis (2)	70.60	1.40
S. parasanguinis (3)	162.30	1.60
Mean±sd	116.93±45.86	1.50±0.10

5.2.4 Quality of DNA sequencing

After generating DNA sequences for the DNA extracts using Illumina sequencing technology, the quality of the sequences was analysed. In the present study, the quality parameter of sequencing was determined using the PHRED quality score, which provides a quality measure of nucleotide bases generated by automated DNA sequencing. PHRED (Phred Quality Score) is a widely adopted method that is used to evaluate the accuracy of nucleotide base calls in DNA sequencing. This method assigns a quality score, known as the "PHRED score," to each base call, with higher scores indicating a higher level of confidence in the accuracy of the call. PHRED scores are computed by taking into account the probability of an incorrect base call. The raw data generated by DNA sequencers provides an estimation of the error probability of

a base call at each position. The PHRED algorithm then converts this error probability into an integer value, referred to as the Q-score (PHRED score). The Q-scores have a logarithmic relationship to the error probability, with a scaling factor of 10. For example, a Q-score of 30 corresponds to an error probability of approximately 0.001(Ewing and Green, 1998).

These scores are crucial in identifying and eliminating low-quality base calls during downstream data analysis. This approach helps to improve the overall accuracy of the final sequence. Additionally, PHRED scores provide an objective measure for filtering low-quality data while retaining high-quality data. This enables the setting of a threshold for quality to retain only high-quality data and eliminate data that may be prone to errors and affect downstream analysis. PHRED quality score is a reliable and well-established method to quantify the accuracy of nucleotide base calls in DNA sequencing (Ewing and Green, 1998). By converting the error probability into an integer value, it allows for the identification of low-quality reads which can be excluded from downstream analysis. This improves the overall accuracy of the final sequence.

In this method, each base is assigned a probability 'score' of how likely the base designation is incorrect (Ewing and Green, 1998; Ruffalo *et al.*, 2012). According to Ewing and Green (1998), PHRED scoring has a high statistical power to discriminate between accurate and inaccurate base-calls, for reading data collected under different chemistries and electrophoretic conditions. In the present study, the PHRED score was mathematically defined as Q which is the log-scaled probability that the base call is incorrect (Ruffalo et al., 2012), as shown in the equation below.

$$Q = -10 \log_{10} P(\text{base is incorrect})$$

Where Q is the PHRED score; P is the estimated error probability of a base designation.

A base call with an error probability of 1/1000 for a given base call is assigned a quality value of 30. A Q value of 30 or greater is considered of high quality and corresponds to low error probabilities (Ewing and Green, 1998). A PHRED score of 30 or greater is thus a high-quality sequence and provides an idea of the degree of quality of sequencing data, in terms of the percentage of the short potentially inaccurate reads. This is called the Q30%. In the present study, the Q 30% and raw reads for each sample are summarised in Table 5.3 The values are for the Forward (F) reads and reverse (R) reads of the paired ends. The R-value is always lower than the F accuracy. These values were obtained by using the High-Throughput Quality Control (HTQC), a program tool kit for sequence reads quality control as described in Yang *et al.* (2013). The reference strain (WT) had higher reads (1,923,378) than *S. parasanguinis* colonies from co-cultures with TIGR4 WT strain (mean 1754498.67±68153.67). F-Reads Q30% were ≥ 95.5 % for both reference strain (WT) and sample *S. parasanguinis*. The R-reads ranged from 74.8 to 75.1 (mean 75%) for the samples, which was slightly lower than 75.4 % for the reference strain (WT). R-reads Q30% were lower than F-Reads Q30% as expected.

Table 5.2: Read Statistics: Forward (F) reads and reverse (R) reads of the paired ends of the DNA samples processed by HTQC

Sample	Reads	F-Reads Q30%	R-reads Q30%
Reference strain (WT)	1,923,378	95.6 %	75.4 %
S. parasanguinis R_1	1,688,658	95.7 %	75.1 %
S. parasanguinis R_2	1,824,751	95.5 %	75.1 %
S. parasanguinis R_3	1,750,087	95.8 %	74.8 %
Mean±sd	1754498.67±68153.67	0.96	0.75

Another indication of quality is the Phred values across the whole length of a read. This is shown in the boxplots of per-base quality statistics for the WT forward and reverse reads (Figure 5.4). The boxplots were generated using the FastQC program, which was developed to

provide a simple way to do some quality control checks on raw sequence data coming from high throughput sequencing pipelines. Through its graphical presentation, it provides a modular set of analyses that are easy to visualize any problems in the data that one should beware of before doing any further analysis (Andrews, 2010). Overall, the reads from this trace are excellent; the reverse is always a bit worse. The reverse read has poorer quality base. This is usually the case, at least for illumina. This is because the reverse reads are generated after the forward reads. The other samples gave very similar plots.

The y-axis on the boxplots shows the base quality score Q_b while the x-axis is the base position in each read. The higher the Q_b score the better the base call. The background of the graph divides the y-axis into excellent quality calls (green), calls of reasonable quality (orange), and calls of poor quality (red). The quality of calls on most platforms degrade as the run progresses, so it is common to see base calls falling into the orange area towards the end of a read. It should be mentioned that there are several different ways to encode a quality score in a FastQ file. FastQC attempts to automatically determine which encoding method was used, but in some very limited datasets, it may guess this incorrectly (ironically only when your data is

universally very good!). The title of the graph will describe the encoding FastQC thinks your file used.

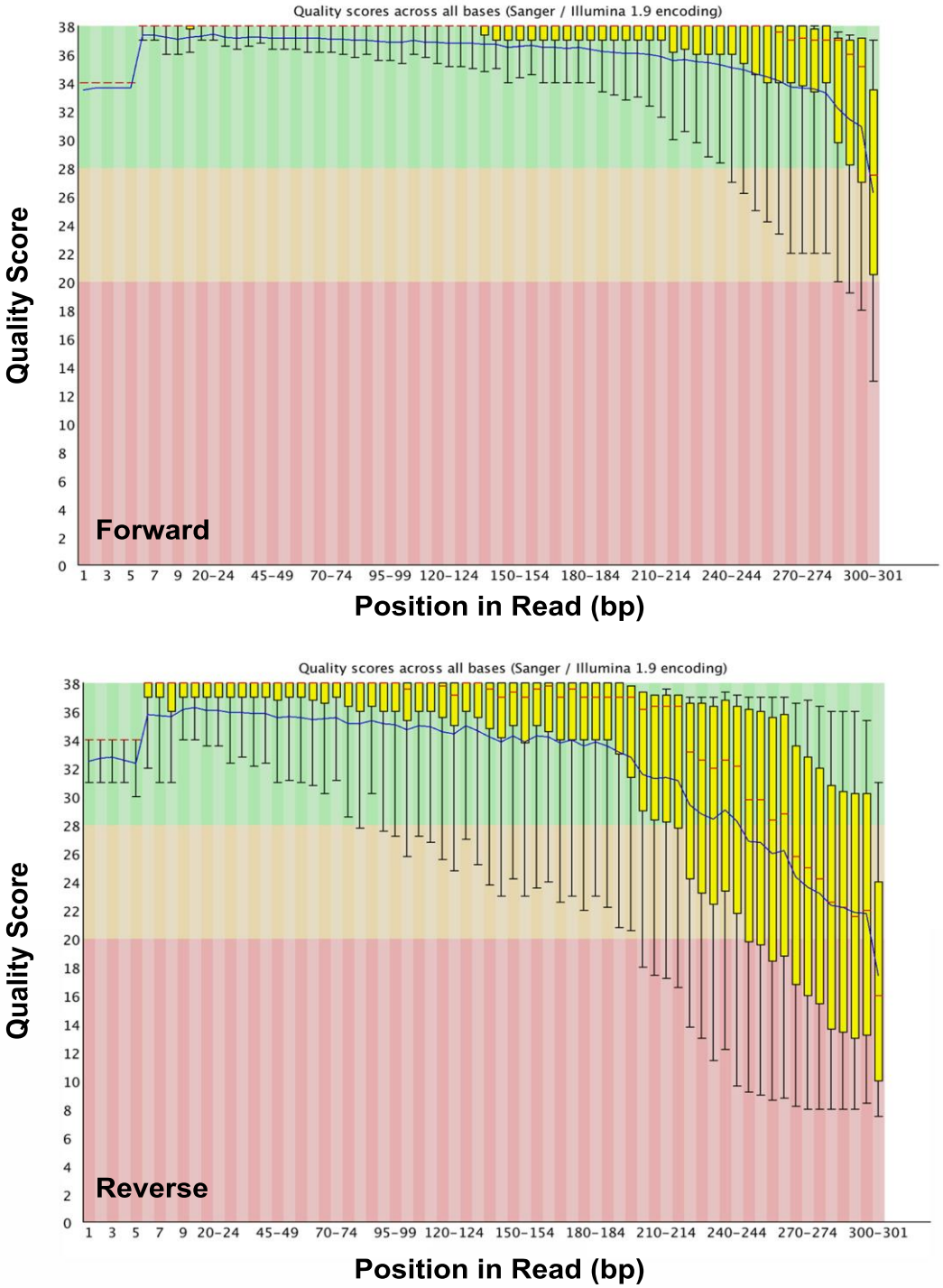

Figure 5-2: Box and whisker plots of per-base quality statistics generated by the FastQC tool (Andrews, 2010) for DNA extracts of *S. parasanguinis* across a random sample of 1,754,498.67 mean reads. The x-axis is the base position in each read, and the y -axis is the base quality score Q_b. The central red line is the median value; the yellow box represents the inter-quartile range (25-75%); the upper and lower whiskers represent 10% and 90% points; the blue line represents the mean base quality.

5.2.5 Checking the Origin of The Sequences

To check the species of bacteria, we used ribosomal multi-locus sequence typing (rMLST) as described by Jolley *et al.* (2012). Rmlst is an advanced method of identifying and characterizing bacterial strains based on the analysis of multiple genetic loci within the ribosome. This technique utilizes PCR amplification and sequencing of several key regions within the ribosome, such as the 16S and 23S rRNA genes, to generate a unique genetic profile for each strain. These profiles are then compared to a comprehensive database of known rMLST profiles to determine the strain's identity and infer any genetic relatedness. One of the main advantages of rMLST over other sequence-based typing methods, such as MLST, is its specificity for the ribosomal genes. This not only enhances the accuracy and reliability of the method, but also enables it to be used for a wide range of applications, such as identifying and tracking the spread of bacterial pathogens and understanding the evolution and diversity of bacterial species (Jolley et al., 2012).

In addition, rMLST is particularly useful for identifying and characterizing bacterial strains from the same species but from different sources. By comparing the rMLST profiles of strains from different origins, it is possible to infer the origin of the bacterial sequences and track down the genetic relatedness of the species (Jolley et al., 2012). This can be especially useful for public health and epidemiological studies. The rMLST is a powerful and versatile technique for studying bacterial genetics and evolution. It's one of the most common methods used in Microbiology laboratory to track bacterial populations and identify outbreaks (Geng *et al.*, 2012).

This approach integrates microbial genealogy and typing through indexing of the variation of 53 genes encoding bacterial ribosome protein subunits (*rps* genes). The indexing employs carefully chosen and efficiently organized reference sequences to identify gene variants efficiently and rapidly (Jolley et al., 2012). The variation in the *rps* gene is organized in a

curated database of catalogue, and therefore, permits rapid and efficient identification of the phylogenetic position of any bacterial sequence at the domain, phylum, class, order, family, genus, species and strain levels (Jolley et al., 2012). In the present study, the Illumina data from generated from our DNA sample were run on the rMLST. The rMLST identified our generated Illumina sequence data as from *S. parasanguinis.* In the microbiology lab, the organism was originally called *S. salivarius*. However, *S. parasanguinis* and *S. salivarius* are both closely related oral streptococci. They are both commensal species commonly found in the human oral cavity and digestive tract, though both can cause human infections such as meningitis, endocarditis, and bacteraemia (Geng *et al.*, 2012).

5.2.6 Assembly of the WT Sequences to Produce A 'Reference'

To identify any base changes in the resistant mutants, we needed to construct a reference assembly from the WT sequences. This was performed using a program called SPAdes (St. Petersburg genome assembler), an online-based open-source software program (http://bioinf.spbau.ru/spades). SPAdes (St. Petersburg genome assembler) is an open-source, online-based software that is widely employed for genome assembly. It is a robust and highly accurate tool, making it an ideal choice for constructing reference genomes. One of the most significant strengths of SPAdes is its ability to produce highly accurate assemblies, even in the presence of high levels of structural variation or sequencing errors. This is particularly crucial when constructing a reference genome, as the reference must be highly accurate to be useful. Another key advantage of SPAdes is its versatility in handling a wide range of sequencing technologies, including Illumina, PacBio, and Nanopore. This allows for the assembly of genomes from various sequencing platforms, which is useful in a laboratory setting that uses a variety of technologies. Additionally, SPAdes use different k-mer sizes for assembly, which can improve the accuracy of the assembly by capturing information from different parts of the genome.

The software is also equipped to handle large datasets, including high-coverage genome assemblies, which is particularly useful for the assembly of large and complex genomes. Furthermore, it uses a hybrid approach, which combines the advantages of both de novo and reference-based assembly methods, to construct assemblies that are robust to incomplete or fragmented input data. Being an open-source software, SPAdes is freely available to the community, and it comes with the source code which makes it easy to customize, modify, or incorporate into other workflows. Its high accuracy rate, versatility, and ability to handle large datasets and incomplete input data, coupled with being an open-source software, make it a widely used and preferred choice among researchers for genome assembly.

The SPAdes program takes the paired end-reads and assembles them as far as possible. However, assembly of short reads is usually not perfect, as only 'contigs' – long assembled reads are obtained. The 'contigs' are imperfect reads that the program attempt to build into longer pieces, called scaffolds. Briefly, in the present study, the paired end-reads were inputted (using -1 and -2 flags) and adjusted carefully to reduce mismatches and short deletions. The completed genome assemblies were checked for quality by computing some key quality parameters using another software program called QUality ASsessment Tool 5.0.2 (QUAST 5.0.2). The QUAST can evaluate and compare genome assemblies with or without a reference genome. QUAST determines the number of mis-assemblies in a genome assembly, using Plantagora's definition. According to Plantagora's definition, a mis-assembly breakpoint is a position in the assembled contigs, such that the left flanking sequence aligns ≥ 1 kb away from the right flanking sequence on the reference genome.

Alternatively, Plantagora's definition of a mis-assembly breakpoint is the overlap of left and right flanking sequence by ≥ 1 kb, or aligning of the two flaking on opposite strands or different chromosomes (Gurevich *et al.*, 2013). QUAST generates a variety of reports, summary tables and plots that aid scientists to interpret, and visually present their results in their publications

(Gurevich et al., 2013). In the present study, QUAST 5.0.2 was employed to assess the quality of the assembly produced by SPAdes. The basic output stats are shown in Table 5.4. From Table 5.4, the total number of contigs (# contigs) in the assembly that were longer than 500 bp was 68. The total number of contigs (>= 0 bp) was 126, while contigs (>= 25000 or 50000 bp) were 6 each. The largest or longest contig in the assembly was 903364 bp in length. The length of the contigs, equivalent to the total number of bases in the assembly was 2134868 bp. Thus, as the base pair size increases, the number of contigs and length decreases. The total length of a collection of all contigs of that length or longer than covered ≥ 50% of the total base content of the assembly (N50) was 454782 while the length of the contig that contained ≥ 75% of the total base content of the assembly (N75) was 279654. These translated to an L50 (the number of contigs ≥ the N50 length) of 2 and an L75 (the number of contigs ≥ the N75 length) of 3. N50 and L50 were 1.5-fold larger than N75 and L75. Therefore, this was a good statistic of how well the assembly worked is the N50. If the contigs are ordered by length, largest to smallest, the N50 is the length of the contig by which 50% of the assembled nucleotides are reached. Obviously, the bigger this is the better. Here, the N50 is 454782 which was acceptable. The ratio of the total number of G and C nucleotides in the assembly to the total length of the assembly as a percentage (GC %) was 42.07 %. The average number of uncalled bases (N's) and N's per 10,0000 assembly bases (N's per 100 kbp) were both zero, indicating no mismatches in our genome assemblies.

Table 5.3: QUAST statistics of the number of genome mis-assemblies without a reference genome

Statistics without reference	contigs
# contigs	68
# contigs (>= 0 bp)	126
# contigs (>= 1000 bp)	14
# contigs (>= 5000 bp)	7
# contigs (>= 10000 bp)	7
# contigs (>= 25000 bp)	6
# contigs (>= 50000 bp)	6
Largest contig	903364
Total length	2134868
Total length (>= 0 bp)	2159562
Total length (>= 1000 bp)	2104472
Total length (>= 5000 bp)	2093749
Total length (>= 10000 bp)	2093749
Total length (>= 25000 bp)	2072939
Total length (>= 50000 bp)	2072939
N50	454782
N75	279654
L50	2
L75	3
GC (%)	42.07
Mismatches	
# N's	0
# N's per 100 kbp	0

In the above QUAST statistics, the "average" contig length for a new genome assembly is measured by N50, which is neither the real average nor median value (Heng, 2020). Since the length of N50 correlates positively with better contiguity, it can be similarly defined as Nx, where contigs of ≥ Nx in length covers x% of the assembly (Heng, 2020). This can be presented as Nx curve plots, where Nx is plotted as a function of x, where x ranges from 0 to 100 (percentage of the total assembly length as the x axis). Figure 5.5 which was generated from the QUAST software shows the Nx curve plots, where the y-axis is the lengths of each contig while the x-axis is the percentage of the total assembly length. The red curve shows good assembly, because it has better contiguity, since it covers more than 40% of the assembly.

Furthermore, the Nx curve is good because it is “higher”, giving a larger Area Under the Curve (AUC). In this case, the AUC for the Nx curve (AUNx) can be used as a measurement of contiguity (Heng, 2020).

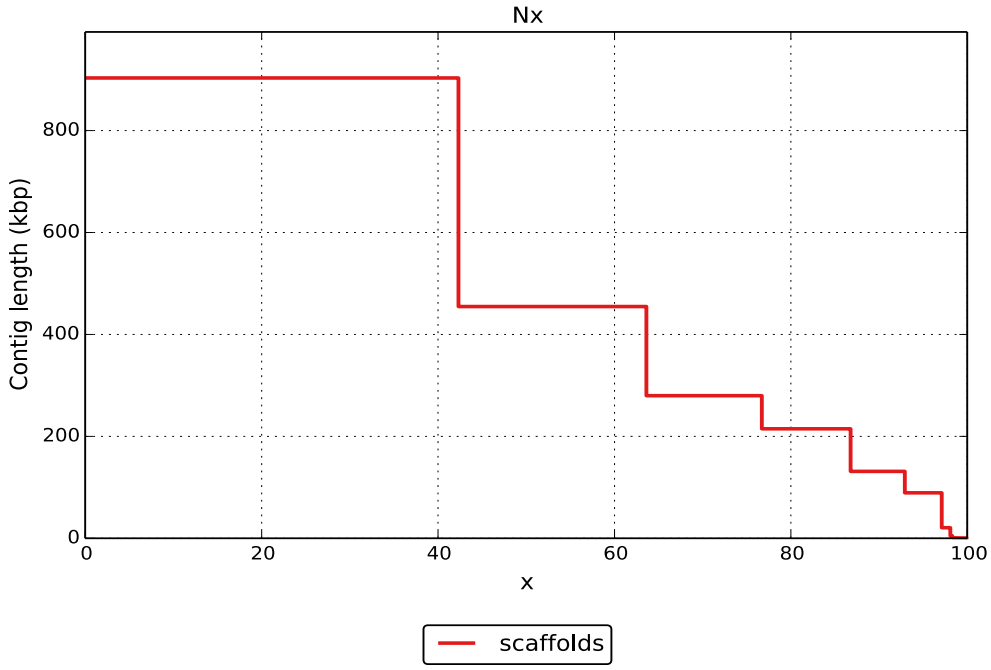

Figure 5-3: Nx curve of contig length (kbp) against x (%) of the assembly cover. In the Nx curve, a single contig covers more than 40% of the assembly, which indicates good contiguity.

Figure 5.6 below shows the number of bases (y-axis) versus coverage values. It shows a normal distribution of bases across coverage values ranging from 0 to 1000. From the curve, approximately 0 bases, 10,000 bases and 600 bases are had coverage values of 250, 500 and 750, respectively. The peak number of bases was approximately 12,400 with coverage value of approximately 550. The distribution normal and therefore, good.

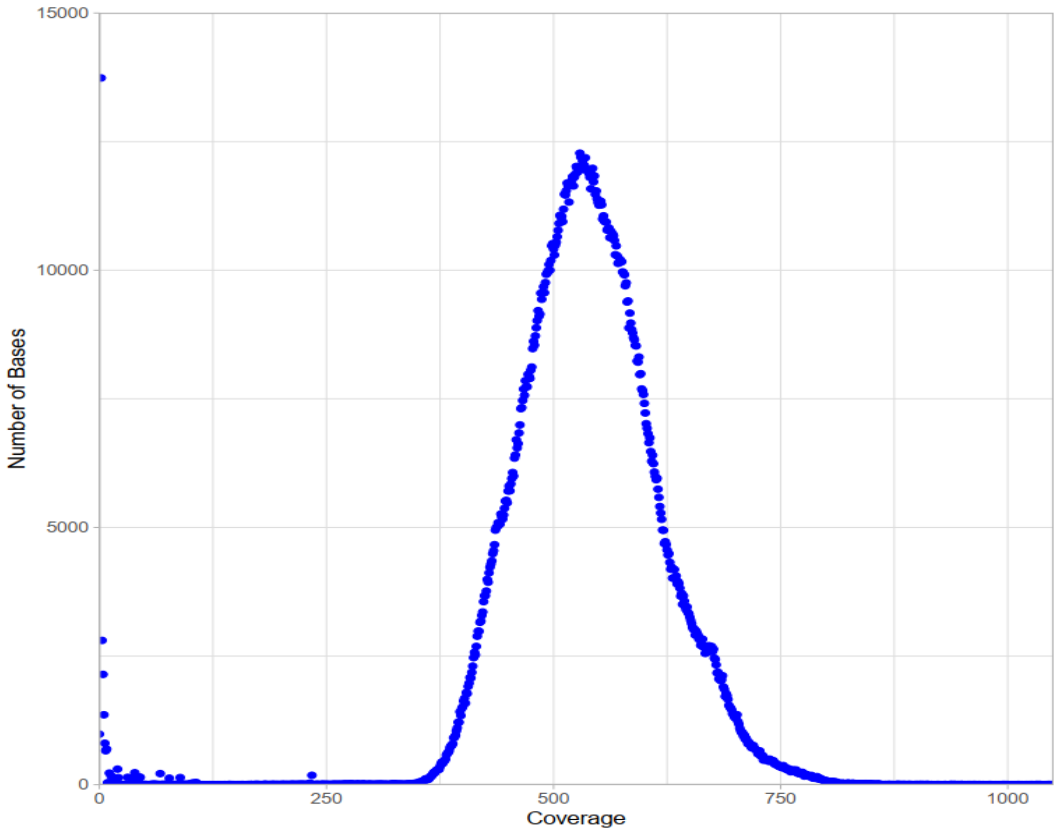

Figure 5-4: The distribution of the number of bases (y-axis) versus coverage values. This curve shows a normal distribution of the number of bases across the coverage value range of 0-1000.

Another useful quality metric is the amount of coverage of the assembly provided by the reads. This is the number of short reads containing a particular base. This proportion should be reasonably high as there will be some errors in the Illumina paired-end reads and therefore, having the same base in multiple sequences allows the consensus base to be designated. Average coverage for the reads across this assembly was calculated using Brian Bushell's Map (BBMap) short read aligner (Bushell, 2021). The BBMap tool aligns the short reads for DNA- and RNA-sequence data to the reference and then estimates how good the coverage is. The BBMap output in this study is summarised in Table 5.5. In the present study, 125 of the possible 126 contigs were mapped using the BBMap short read aligner. One contigs was excluded because it was too short to be included and had undesirable repeats. The average coverage from the paired reads was 531.422, which was considered markedly high. This indicated that the reference was of very high quality.

Table 5.4: The BBMap output statistics for the short-read alignment.

BBMap Stats	Values/results
Reads	3846756
Mapped reads	3814129
Mapped bases	1147691947
Ref scaffolds	125
Ref bases	2159662
Percent mapped	99.15%
Percent proper pairs	98.12%
Average coverage	531.422*
Average coverage with deletions	530.839
Standard deviation	138.188
Percent scaffolds with any coverage	96.80%
Percent of reference bases covered	99.95%

5.2.7 Annotation

The reference sequence was then annotated using PROKKA (rapid prokaryotic genome annotation) as described by Seemann (2014). PROKKA is regarded as a command-line software tool that aids full annotation of draft genomes of bacteria, archaea and viruses. It generates standard-compliant output files that are amenable to further analysis and viewing by conventional genome browsers (Seemann, 2014). PROKKA, short for rapid prokaryotic genome annotation, is a widely used software for annotating reference sequences. Its popularity is attributed to its combination of speed, accuracy, and comprehensiveness when it comes to identifying and describing different features of the genome, such as genes, tRNAs, rRNAs, CDS, promoters, intergenic regions and regulatory elements.

One of the key strengths of PROKKA is its ability to quickly and accurately annotate a genome, making it an ideal tool for large-scale annotation projects. It uses a combination of different methods such as ab initio predictions, homology-based inferences, and database searches which allows for accurate prediction of the gene content and the related functional roles. The annotation includes functional information, such as the putative function of the encoded proteins, the location of the gene, and the strand on which the gene is encoded. Another

advantage of PROKKA is that it is a cross-platform software, which can run on Windows, MacOS, and Linux, and it can be integrated into various pipelines, making it a versatile tool for annotation. Additionally, it is an open-source software, making it freely available to the community with the source code, which makes it easy to customize, modify, or incorporate it into other workflows. PROKKA can output the annotation in various formats like GenBank, GFF3, and FASTA, providing flexibility in the way the annotation information can be used. Overall, PROKKA is a powerful and efficient tool for annotating reference sequences, and it's widely used in the field of genomics and microbiology because of its speed, accuracy and comprehensiveness, versatility and open-source nature.

In the present study, our reference sequence was annotated using Genbank (https://www.ncbi.nlm.nih.gov/nuccore/NC_015678.1) as the reference genome. Our reference genome is a fully sequenced reference strain *S. parasanguinis* ATCC 15912. For each coding region, the 'addgenes' tool of the PROKKA program was used to add a 'gene' feature while the 'addmrna' tool was used to add an 'mRNA' feature. Several files including an entire GenBank file were generated from the PROKKA program

5.2.8 Comparing the Three Mutants to the Reference Genome

The three mutants of the *S. parasanguinis* were compared with the reference genome using the SNIPPY pipeline. The SNIPPY tool is valuable for rapid bacterial SNP (single-nucleotide polymorphism) and deletions and insertions calling and core genome alignments (Seemann, 2021). This was used with forward (F) and reverse (R) reads for all the three mutants of *S. parasanguinis* and compared to the reference as a fully annotated GenBank file. No genetic variations (zero SNPs) were detected, nor any changes in which a specific nucleotide sequence was present (insertion) or absent (deletion). In addition, the morphology of the three mutants of *S. parasanguinis* remained unchanged as compared to the reference, suggesting no evidence of phase variation.

5.2.9 Discussion

Single nucleotide polymorphism (SNP) is mutations are random changes (gene insertion or deletions) in the nucleotide sequence of a short region of a genome and usually culminate in phenotypic changes of varying degrees depending on the severity and location of the mutation (Watford and Warrington, 2021). Bacteria exposed to external (environmental) factors such as radiation or harmful chemicals and biotoxins, including bacteriocins are susceptible to mutation (Campelo *et al.*, 2020). Mutations in bacteria, induced by external factors are typically adaptive mutations as a response to survival threats (Rosenberg, 1997; Samhita *et al.*, 2020). In the present study, *S. parasanguinis* was co-cultured with TIGR4 strain that produces bacteriocins. The two-peptide bacteriocins initially suppressed the growth of the rival *S. parasanguinis* (first 2 h), which regained its growth afterward (2-6 h). The bacteriocin-resistance phenotype of *S. parasanguinis* was confirmed. The present study tested hypotheses that bacteriocin resistance in *S. parasanguinis* was due to mutations in bacteriocin target genes and/or the acquisition of self- bacteriocin-resistance genes from TIGR4 via horizontal gene transfer.

First, our genomics data obtained was of exceptional quality. The number of bases were distributed normally across the coverage range of 0-1000. Our step-by-step DNA sequencing data analysis and quality control indicate that our extracted DNA products from *S. parasanguinis* was of high quality. Furthermore, the base calling accuracy of our highly pure DNA extracts from *S. parasanguinis*, as measured by the Phred *quality score* (Q score) also demonstrated that our gene sequence data for the target organism was of high quality. The advantage of Phred quality scoring is that it provides base-specific quality scores (Ewing and Green, 1998), which are highly accurate for comparing different sequencing methods as employed in the present study. Q score is helpful for downstream DNA analysis, such as DNA assembly and the detection of SNP (Zhang *et al.*, 2017).

In the present study, the reference strain (wt) had higher reads (1,923,378) than *S. parasanguinis* colonies from co-cultures with TIGR4 WT strain (mean 1754498.67±68153.67). Findings from our genomics analysis of DNA extracted from *S. parasanguinis* showed no evidence of mutations and, therefore, refuted one of our guiding hypotheses. Additionally, no insertions or deletions in the mutants were present.

According to Dalia and Dalia (2019), natural transformation (NT) is a major mechanism of HGT in microbial species. NT entails microbial acquisition of free DNA, usually double-stranded DNA (dsDNA), from their growth environment by translocation of one of the strands (single-stranded DNA (ssDNA)) into the cytoplasm and subsequently integrate the ssDNA into their genome by homologous recombination. This phenomenon is widely implicated in the widespread of antibiotic-resistance genes and virulence factors in pathogenic bacteria, hence causing both genetic and epigenetic inheritance of resistance traits (Dalia and Dalia, 2019). Therefore, NT could also be responsible for the acquisition or spread of bacteriocin-resistance genes by the *S. parasanguinis*, which eventually developed resistance to the bacteriocin from the TIGR4 WT strain.

However, while HGT by NT promotes both genetic and epigenetic inheritance of traits in bacteria, no significant genetic changes were detected in our target bacteriocin-resistant *S. parasanguinis* species co-cultured with TIGR4 WT strain. Epigenetic mechanisms effects inheritable trait changes by working at the interface of genetic and environmental factors where they induced stable changes in gene expression levels by alterations in the chromosomal superstructure in which DNA is packaged, without interfering with the structure of the DNA sequence (Al Aboud *et al.,* 2021). This indicated that the putative HGT by NT in our strain promoted epigenetic (but not genetic) changes that promoted the expression of the bacteriocin-resistant trait in *S. parasanguinis.*

Indeed, the bacterial genome is susceptible to epigenetic changes. Epigenetic information superimposed over the nucleotide sequence of genome, provides signals for epigenetic changes by controlling DNA–protein interactions. Therefore, although epigenetic mechanisms do not directly alter the DNA sequence, they can regulate gene expression through chemical modifications of DNA bases and changes to the chromosomal superstructure packed with the actual DNA (Al Aboud et al., 2021). Epigenetic changes may result in heritable phenotypic changes in gene expression not substantively encoded by the DNA sequence (Jin *et al.*, 2011). Basically, epigenetic modifications occur in the absence of a mutation (Sánchez-Romero and Casadesús, 2020). The main mechanism of epigenetic changes and signalling is through chemical modifications of DNA bases, typically by DNA methylation.DNA methylation is an epigenetic mark characterised by the covalent transfer of a methyl group (CH_3-), to the C-5 position of the cytosine ring of a DNA catalysed by DNA methyltransferases (DNMTs) (Jin et al., 2011). In bacteria, epigenetic changes by DNA methylation are useful as they play essential role in genome defence, chromosomal replication and segregation, cell-cycle arrest, DNA damage repair, transcriptional regulation and antibiotic resistance mechanism. For example, exposure of bacteria to antimicrobial compounds can result in DNA methylation pattern changes, which result in transient and fast-appearing adaptive antibiotic resistance (AdR) phenotypes, such as strains overexpressing efflux pumps (Papaleo et al., 2022).

In most bacteria species, DNA methylation provides a mechanism for reversible phase-variation (switching) of gene expression, which produces phenotypic cell variants without substantive changes in the DNA (Sánchez-Romero and Casadesús, 2020). This phenomenon is likely to have occurred in our bacteriocin-resistant *S. parasanguinis*, where the bacteria was exhibited bacteriocin resistance occurred in the absence of mutation.

However, our hypothesis regarding probable gene mutation cannot be refuted entirely as there could be several factors in our analysis that could explain the undetected mutations in our test

bacteriocin-resistance *S. parasanguinis*. Large-scale genomic inversion involves large portions of DNA in the range 10,000 - 5,000,000 bases long. This large-scale inversion cannot be picked up by short reads and therefore, no mutation can be detected. The highly likely reasons as to why the putative mutations were not detected in our organism could be large-scale genomic (mutation) inversion and epigenetic changes (as discussed above). However, this could be detected using long-read sequencing technologies such as PacBio or Nanopore, which generate reads in excess of 10 kb, unlike Illumina's NovaSeq (600 bases) (Amarasinghe et al., 2020).

5.2.10 Conclusions

The present study successfully extracted high-quality DNA from bacteriocin-resistant and WT *S. parasanguinis*. The DNA genome sequencing data for the samples was of exceptionally high quality. However, no evidence of mutation was detected in bacteriocin-resistant *S. parasanguinis*, though epigenetic changes could have played a major role in the expression of the bacteriocin resistance trait. Mutations might have occurred but involving large-scale genomic inversion, which could not be detected by short reads. Therefore, further work is warranted to re-evaluate our DNA sequence using long-read sequencing technologies such as PacBio or Nanopore. In addition, the study conducted an examination of mutation and horizontal gene transfer as potential mechanisms for the development of resistance to bacteriocins in the bacterium *S. parasanguinis*. bacteriocins are small peptides that are produced by bacteria as a defence mechanism against other bacteria. The study co-cultured *S. parasanguinis* with a strain that produces bacteriocins and observed that the growth of *S. parasanguinis* was initially suppressed but then regained, indicating the development of resistance. The study then used genomics analysis to test the hypotheses that resistance in *S. parasanguinis* was due to mutations in bacteriocin target genes or the acquisition of self-bacteriocin-resistance genes via horizontal gene transfer. The genomics data obtained was of high quality, and the analysis showed no evidence of mutations in the bacteriocin-resistant

strain of *S. parasanguinis*. Additionally, no insertions or deletions were present, refuting the hypothesis that resistance was due to mutations. The study then suggested that the resistance was likely due to horizontal gene transfer via natural transformation, a process in which bacteria acquire free DNA from their environment and integrate it into their genome by homologous recombination. The study proposed that natural transformation promoted epigenetic changes in gene expression rather than genetic changes, leading to the expression of the bacteriocin-resistant trait.

CHAPTER 6: The Role of Metal Ions in Pneumococcal Bacteriocin Inhibition

6.1 Introduction

S. pneumoniae is a causative agent leading to most hospitalization infections in the children population in the United States. The bacterium lives as commensal organisms in the nasopharynx cavity of humans, where concentrations of metal ions are scarce *(Burcham et al., 2020)*. Metal ions availability is essential for the microbe's cellular metabolism and other physiological activities. However, the human immune system has antimicrobial response attacks in preventing the bacteria from obtaining their nutrient demands *(Burcham et al., 2020)*. The bacteria have evolved mechanisms of obtaining these ions from their host system for survival to counter the limited metal ions concentration in the nasopharynx region. Metal ions required by the *Streptococcus* bacteria include zinc, cobalt, and nickel.

Understanding the antimicrobial resistance mechanism can be used to understand their physiological functions in developing medical procedures for managing and treating infections caused by bacteria. According to *Burcham and colleagues*, metal homeostasis has been projected as a therapeutic strategy for understanding the roles of metal ions in the growth and survival of *S. pneumoniae.* Zinc metal has been linked to the biofilm formation of the bacteria, invasion, and adherence mechanisms of the microbe. Excess zinc metal ions availability is linked to the regulation of the genetic locus of the bacteria *(Burcham et al., 2020)*. The additional gene locus regulates the transport system of the cellular metabolism functions of the bacteria.

The transport pathway also enables the bacteria to adapt to oxidative stress, metal ions acquisition and absorption, and metabolism of carbohydrates from the host system by inhibiting the phosphofructokinase and glyceraldehyde-3-phosphate-dehydrogenase (Burcham et al.,

2020). Metal ions of zinc, iron, and manganese act as antioxidants to react with the hydroxyl ions to form hydroxy radicals used by the bacteria in their antimicrobial resistance properties. Mutated genes thus become resistant to drugs over time. Loss of the gene loci involved in metal ions used in the metabolic functions of the *S. pneumoniae* can be initiated as a therapeutic mechanism of altering their genomics for antibacterial drug elimination.

Metal ions are necessary for bacterial growth and host infection. Approximately one quarter to one-third of proteins need a metal ion cofactor to perform biological functions, including catalysis, metabolic regulation, cell division, and structural composition (Cao *et al.*, 2018; Pernil and Schleiff, 2019; Zhang and Zheng, 2020). For instance, manganese (Mn) serves as a co-factor for many enzymes involved in essential cellular biochemical processes including phosphorylation, hydrolysis, carbon metabolism, decarboxylation, and oxidative stress (Eijkelkamp *et al.*, 2014; Simons et al., 2020). Divalent metal ions are of particular interest in the microbiology of pathogenic bacteria as they have been shown to aid the invasion of pathogenic bacteria into their hosts for successful infection (Eijkelkamp et al., 2014; Weiss and Carver, 2018).

Metal ion transporters are a diverse group of metalloproteins embedded in the plasma or organellar membrane, where they function to transport specific metal ions in and out of the cytosol to ensure their optimal ionic concentrations in different cellular compartments. Cytosolic metal ions may alter the antimicrobial properties of protein bacteriocins. While AMPs are produced by the host cell to inhibit pathogenic bacteria, bacteriocins, on the other hand, are produced by bacteria to inhibit growth activities of similar or closely related rival bacterial strains (Simons et al., 2020). The outer membrane (OM) of bacteria is known to block the entry of antibiotics such as vancomycin, hence the observed resistance of Gram-negative bacteria against the antibiotic. However, iron transporters can aid the importation of bacteriocins across the OM, hence promoting the inhibition potency of bacteriocins (White *et*

al., 2017). Therefore, bacteria cultured in growth media containing low metal ion concentration may upregulate the activity of ion transporter proteins in the importation of bacteriocins across OM, hence increasing the inhibition of rival strains. However, metal ions do not always exert the same effect on the antimicrobial properties of bacteriocins and these may vary from one bacterium to another. For instance, Fe^{2+} and Mn^{2+} have been demonstrated to enhance while Cu^{2+}, K^{+}, Ca^{2+}, Zn^{2+}, Mg^{2+}, and NaCl inhibit antimicrobial properties of bacteriocins from lactic acid bacteria (LAB) (Matevosyan *et al.*, 2019; Zhang *et al.*, 2018).

Fe^{2+} and Mn^{2+} are essential for bacterial survival and virulence due to their critical role in catalysis, infection, and biofilm formation (Cao et al., 2018). *S. pneumoniae* was demonstrated to possess the ability to grow in a metal ion-depleted medium, where bacterial Fe^{2+} concentration increased with increasing depletion of Mn^{2+}, indicating a compensatory effect of Fe^{2+} for Mn^{2+}. In this respect, the growth of *S. pneumoniae* in the metal ion-depleted medium increased with increasing concentrations of Fe^{2+} (0, 2, 5, 10, 25, and 50 μM) (Cao et al., 2018). Several iron uptake systems have been discovered in *S. pneumoniae.* Three loci have been discovered: *piuBCDA, piaABCD,* and *pitADBC* (Honsa et al., 2013). Within these loci, specific iron transporters are encoded that have been demonstrated to mediate Fe^{3+} uptake (Brown et al., 2002) (Brown J et al., 2001). Fe^{3+} has also been demonstrated to partially reverse the growth defect seen in iron-depleted media for single mutants in these loci. Fe^{2+} also had a similar role, although the selectivity for Fe^{3+}or Fe^{2+} is not clear. The relevant transporters seem to be ABC iron transporters.

In the present study, we investigated the growth of *S. pneumoniae* in a metal ion-deficient medium, as well as the effects of metal cation deficiency on bacteriocin production and the ability to kill related streptococcal species. The tube inhibition assay was performed to investigate changes in inhibition ability in the bacterium in a metal ion-deficient medium. Investigation of metal ion dependency of bacteriocin inhibition in the cation depleted medium

was of interest because many bacteriocins have been found to gain entry to cells using metal ion transporters, particularly iron transporters. Thus, we hypothesised that reducing metal ion concentrations in the medium could lead to upregulation of the relevant transporter. If this was used as a target by the pneumococcal bacteriocins, then this could lead to enhanced inhibition. Alternatively, metal ions could play a significant role in the production or inhibition ability of pneumococcal bacteriocins. The results presented in this chapter examined these hypotheses.

6.2 Results

We set out to determine the effect of different metal cations on the ability of *S. pneumoniae* bacteriocins to produce inhibition. We chose to investigate the effect of altering the concentrations of different metal cations, iron, manganese, magnesium and calcium. We chose these metal ions as transporters since iron and manganese are essential for pneumococcal growth and virulence potential, and calcium and magnesium have many effects on protein function, as outlined in the introduction.

We wished to grow the different bacterial strains in the metal ion-depleted medium. Firstly, we determined the growth of the pneumococcal strain TIGR4 in a metal ion-depleted medium and with various ions added back to the medium. In these experiments, it is hypothesized that the growth curves for TIGR4 WT in the metal ion-deficient medium supplemented with Fe^{3+} (100, 200, 500 and 1000 μM) and Mn^{2+} (100, 200, 300, and 500 μM) concentration (showed progressive exponential growth and that addition of Fe^{3+} and Mn^{2+} at any concentration (2, 5, 10, or 50 μM) did significantly alter the rate of growth. Even supplementation of the ion deficient medium with calcium or magnesium did not have some marginal effect on bacterial growth. Separate experiments showed the TIGR4 Blp KO and *S. parasanguinis* strains also could grow satisfactorily in the metal ion-depleted medium.

6.2.1 Co-culture of TIGR4 Strain and Different Closely Related Pneumococci in Full Media

To determine whether the reporter strain, *S. parasanguinis*, is killed by the bacteriocins produced by TIGR4 WT, the producer strain, at different metal ion concentrations, we co-cultured the organisms in BHI full media. Figure 6.1 shows that the bacteriocins produced by TIGR4 WT have limited the growth of *S. parasanguinis* at 1-2 hours, and *S. parasanguinis* has reached more than 10^5 afterward when measured on an optochin agar plate. The data shows that the TIGR4 WT strain can produce immediate inhibition of *S. parasanguinis* in the BHI medium, but with the recovery of the target *S. parasanguinis* strain at later time points. This shows that under these conditions, TIGR4 WT limited the growth of *S. parasanguinis* for two hours, and thereafter, *S. parasanguinis* maintained growth at 10^4 cfu/ml when measured on an optochin agar plate. Similar results were observed with the kanamycin-resistant TIGR4 Blp KO strain (Figure 6.1). No inhibition of the TIGR4 Blp KO strain was seen in the metal ion-depleted medium. However, when Fe and Mn is added back to the ion-depleted media, inhibition is started at two hours and continued for up to six hours. Similar results were noted for the organism *S. salivarius*, were inhibition was initiated at two hours and persist for up to six hours. Values represent the means of triplicate results of CFUs as shown in figures (6.3 to 6.18).

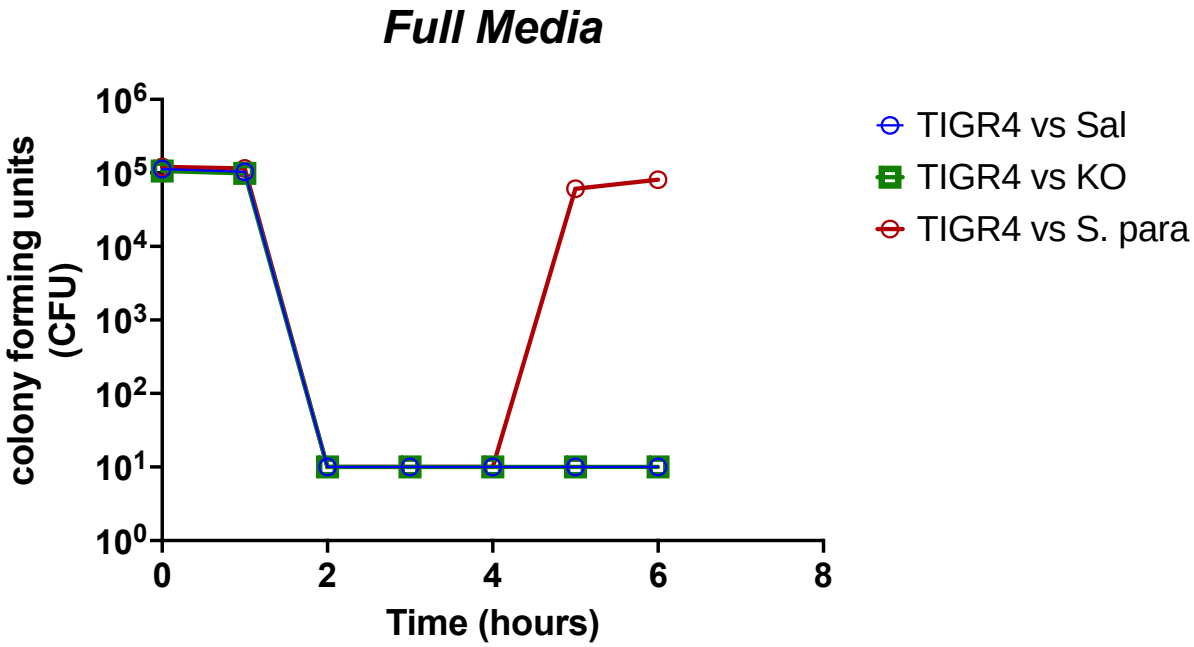

Figure 6-1: Inhibition assay on co-culture of producer TIGR4 WT strain and the different *pneumococci* strains. Red line represents results for target optochin resistant *S. parasanguinis* cells. Blue line represents results for target optochin resistant *S. salivarius*. Green line represents results for kanamycin resistant *TIGR4 KO* strain. Figures represent co-cultures in BHI full media. Values represent the means of CFUs obtained from triplicate cultures plotted against time (hours). Measurements showed less than 1% differences. Error bars are shown on the graph but it does not appear because the three readings are close to each other which indicates good handling technique.

6.2.2 Co-culture of TIGR4 strain and Streptococci Species in Metal Ions Depleted Media.

To determine growth of the TIGR4 strain and three other bacteriocins reporter bacterial strains, co-cultures of *TIGR4* with *S. salivarius*, *TIGR4* with *S. S. parasanguinis*, and *TIGR4* with *TIGR4 KO* were produced in three different tubes filled with BHI metal ions depleted media (Figure 6.2). There was no bacteriocin activity produced by TIGR4 against any of the tested reporter strains.

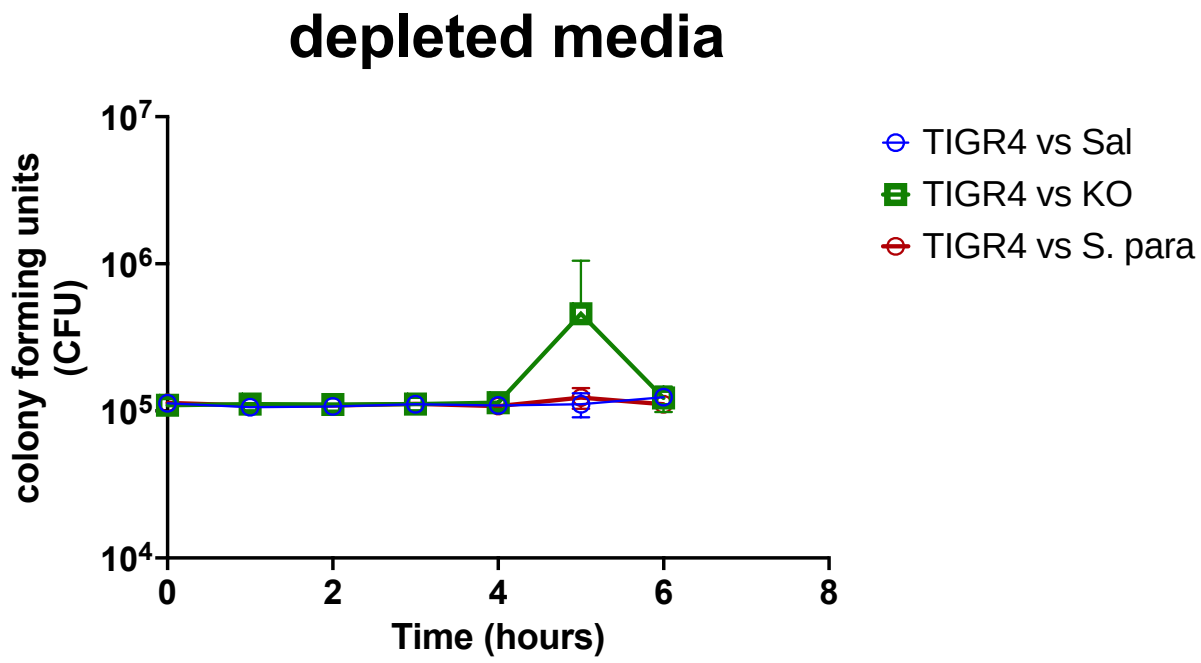

Figure 6-2: Co-culture of TIGR4 strain and Streptococci species in metal ions depleted media. . Blue line represents inhibition assay on co-culture of producer TIGR4 WT strain and the target kanamycin-resistant TIGR4 Blp KO strain. Red line represents inhibition assay on co-culture of producer TIGR4 WT and the target optochin resistant *S. parasanguinis.* Blue line represents inhibition assay on co-culture of producer TIGR4 WT and the target optochin resistant *S. salivarius*. Figure represents co-cultures in three metal ion-depleted BHI medium. Values represent the means of CFUs obtained from triplicate cultures plotted against time (hours). Measurements showed less than 1% differences. The error bars are shown in the graphs and readings are mean of triplicate results.

6.2.3 Co-culture of TIGR4 and Closely Related Streptococcus Species at Different Concentrations of Ca^{2+} ions

To determine whether the addition of Ca^{2+} influences the inhibition activity of bacteriocins secreted by the producer TIGR4 WT strain, we cultured the organism in metal ion-depleted BHI media supplemented with different concentrations of Ca^{2+}. Figure 6.3 shows that the growth curves for co-culture of TIGR4 WT and *S. parasanguinis, TIGR4 WT and S. salivarius,* and *TIGR4* and *TIGR4 KO,* in metal ion-depleted BHI medium supplemented with 100µM $CaCl_2$. *S. parasanguinis* maintained the growth at 10^4-10^5 when measured on an optochin agar plate and therefore, TIGR4 WT cannot kill *S. parasanguinis* in metal ion-depleted BHI medium supplemented with 100µM $CaCl_2$. Similar results were obtained with higher concentrations of

$CaCl_2$ (200, 500, and 1000μM) and for the co-cultures of TIGR4 WT with *S. salivarius* or TIGR4 KO (Figure 6.4, 6.5 and 6.6). Values represent the means of triplicate readings of CFUs. Previous work on the impact of metal ions in the growth of pneumococcus species has shown that increasing concentrations reduces growth opportunities. Pneumococcus species have shown a decreased ability to grow in higher concentrations of Ca^{2+}. Higher concentrations limit the environment's optimum conditions for the species' growth. Figure 6.3 shows that at 100um Ca^{2+} concentration, growth occurs to the optimum OD_{600nm} in 0 to 6hours. Higher concentrations, as shown in Figures 6.4 through 6.7 recorded growth from hours 4-6. The growth patterns under the four concentrations of Ca^{2+} for the three species *TIGR4 and S. Salivarius, TIGR4 vs KO and TIGR4 vs S. S. parasanguinis* under study recorded a similar growth trend with approximately 1% differences.

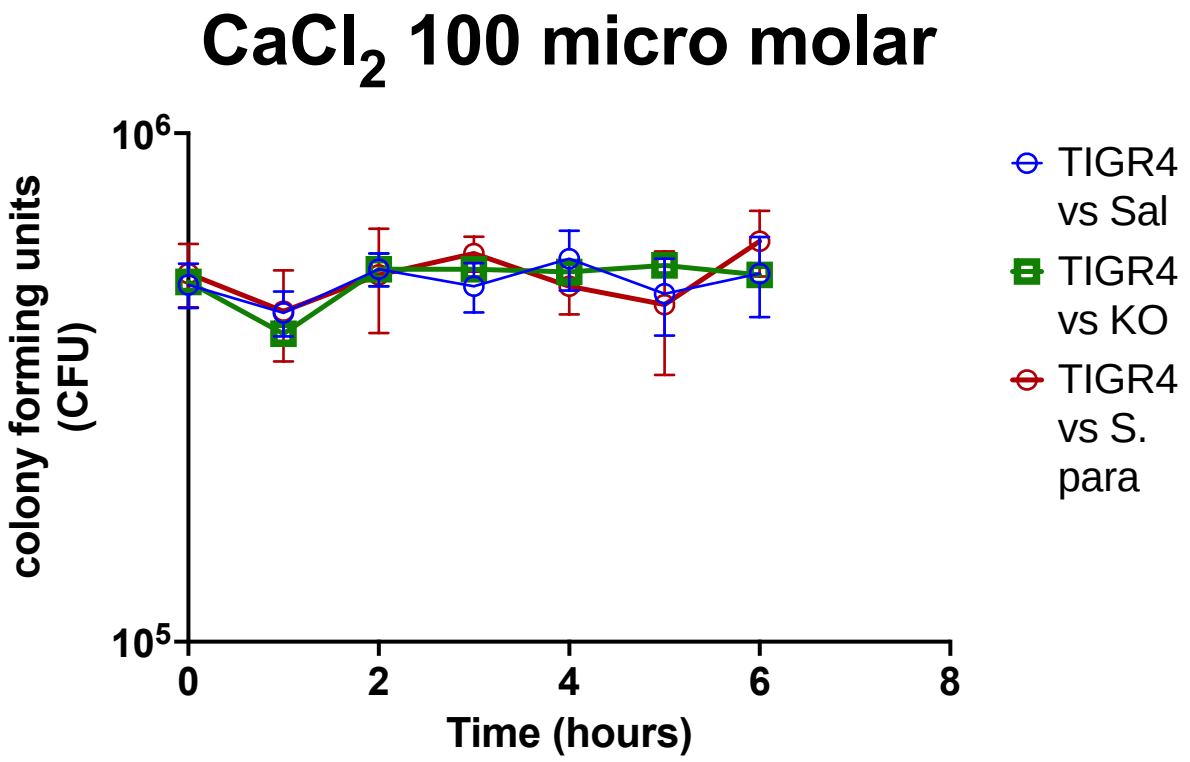

Figure 6-3: Co-culture of TIGR4 and closely related streptococcus species in metal ion-depleted BHI medium supplemented with $CaCl_2$ 100µM . Red line represents inhibition assay on co-culture of producer TIGR4 WT and the target optochin resistant *S. parasanguinis*. Blue line represents inhibition assay on co-culture of producer TIGR4 WT and the target optochin resistant *S. salivarius*. Values represent the means of CFUs obtained from triplicate cultures plotted against time (hours). Measurements showed less than 1% differences. The error bars are shown in the graphs and readings are mean of three.

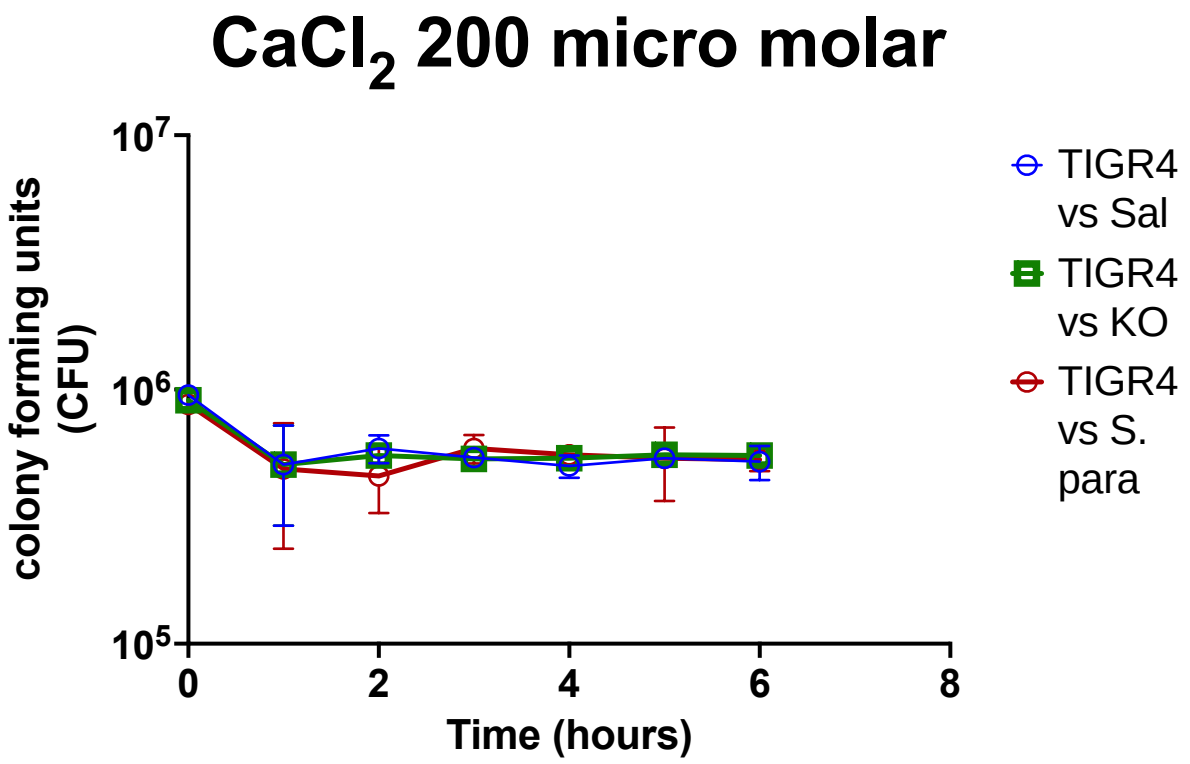

Figure 6-4: Co-culture of TIGR4 and closely related streptococcus species in metal ion-depleted BHI medium supplemented with $CaCl_2$ 200μM. Red line represents inhibition assay on co-culture of producer TIGR4 WT and the target optochin resistant *S. parasanguinis*. Blue line represents inhibition assay on co-culture of producer TIGR4 WT and the target optochin resistant *S. salivarius*. Values represent the means of CFUs obtained from triplicate cultures plotted against time (h). Measurements showed less than 1% differences. The error bars are shown in the graphs and readings are mean of three.

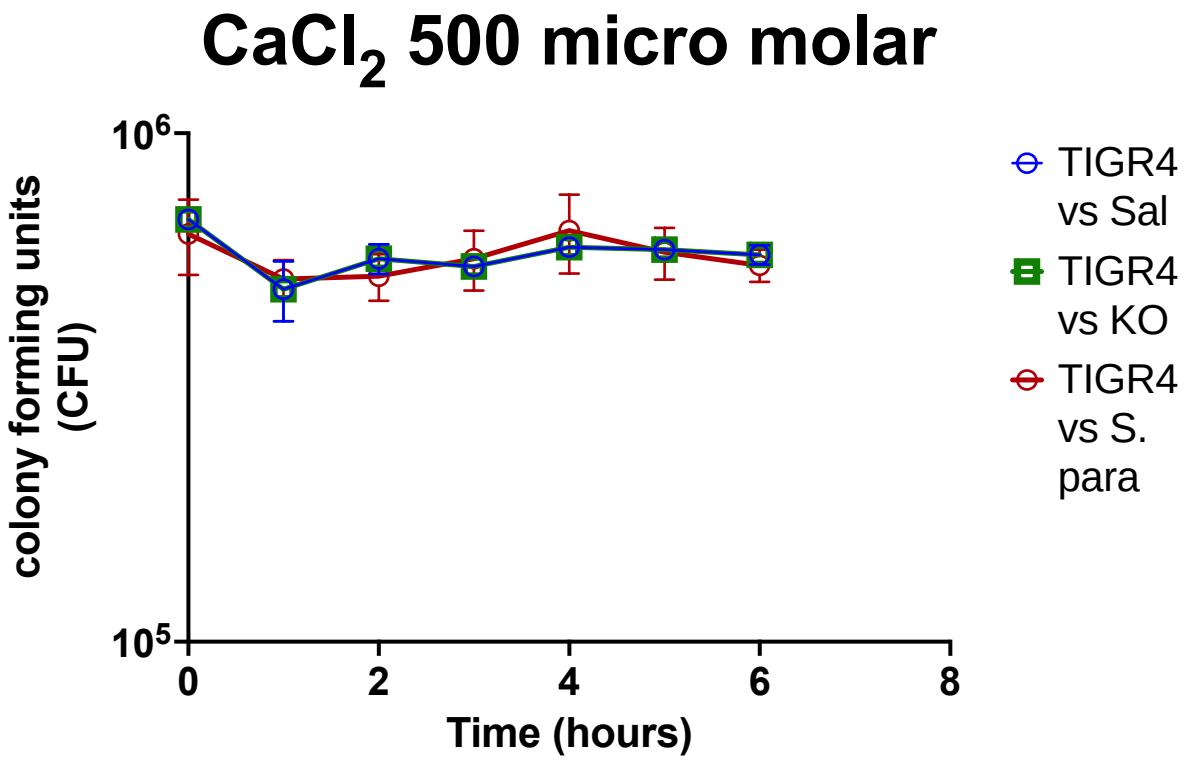

Figure 6-5: Co-culture of TIGR4 and closely related streptococcus species in metal ion-depleted BHI medium supplemented with $CaCl_2$ 500µM . Red line represents inhibition assay on co-culture of producer TIGR4 WT and the target optochin resistant *S. S. parasanguinis*. Blue line represents inhibition assay on co-culture of producer TIGR4 WT and the target optochin resistant *S. salivarius*. Values represent the means of CFUs obtained from triplicate cultures plotted against time (h). Measurements showed less than 1% differences. The error bars are shown in the graphs and readings are mean of three.

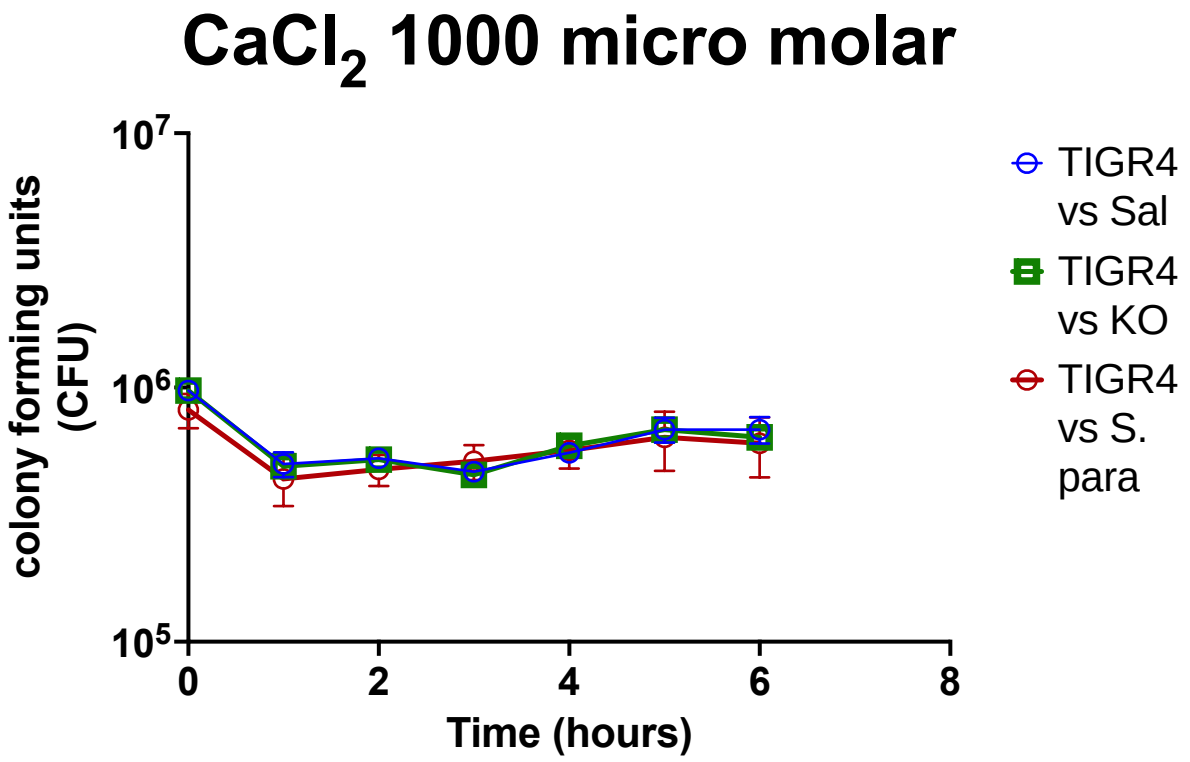

Figure 6-6: Co-culture of TIGR4 and closely related streptococcus species in metal ion-depleted BHI medium supplemented with $CaCl_2$ 1000μM. Red line represents inhibition assay on co-culture of producer TIGR4 WT and the target optochin resistant *S. parasanguinis.* Blue line represents inhibition assay on co-culture of producer TIGR4 WT and the target optochin resistant *S. salivarius*. Values represent the means of CFUs obtained from triplicate cultures plotted against time (h). Measurements showed less than 1% differences. The error bars are shown in the graphs and readings are mean of three.

6.2.4 Co-culture of TIGR4 strain and closely related Streptococcus species at different Mg^{2+} concentration

To determine whether the addition of Mg^{2+} influenced the inhibition achieved by bacteriocins of the producer TIGR4 WT strain, we cultured the organism in metal ion-depleted BHI medium supplemented with different concentrations of Mg^{2+} (100, 200, 300 and 500 μM). Figure 6.7 shows the growth curves for co-culture of TIGR4 WT strain vs *S. parasanguinis, TIGR4 vs S. salivarius, and TIGR4 vs TIGR4 KO* in metal ion-depleted BHI medium supplemented with 100μM $MgCl_2$. *S. parasanguinis* maintained the growth at 10^4-10^5 when measured on an optochin agar plate. This data confirms that the TIGR4 WT cannot kill *S. parasanguinis* in metal ion-depleted BHI medium supplemented with 100μM $MgCl_2$. Similar results were noted

when metal ion-depleted BHI medium was supplemented with higher concentrations Mg^{2+} (200, 300, and 500 μM) and for TIGR4 vs *S. salivarius*, and TIGR4 vs TIGR4 KO as shown in Figures 6.8-6.10. Values represent the means of triplicate readings of CFUs.

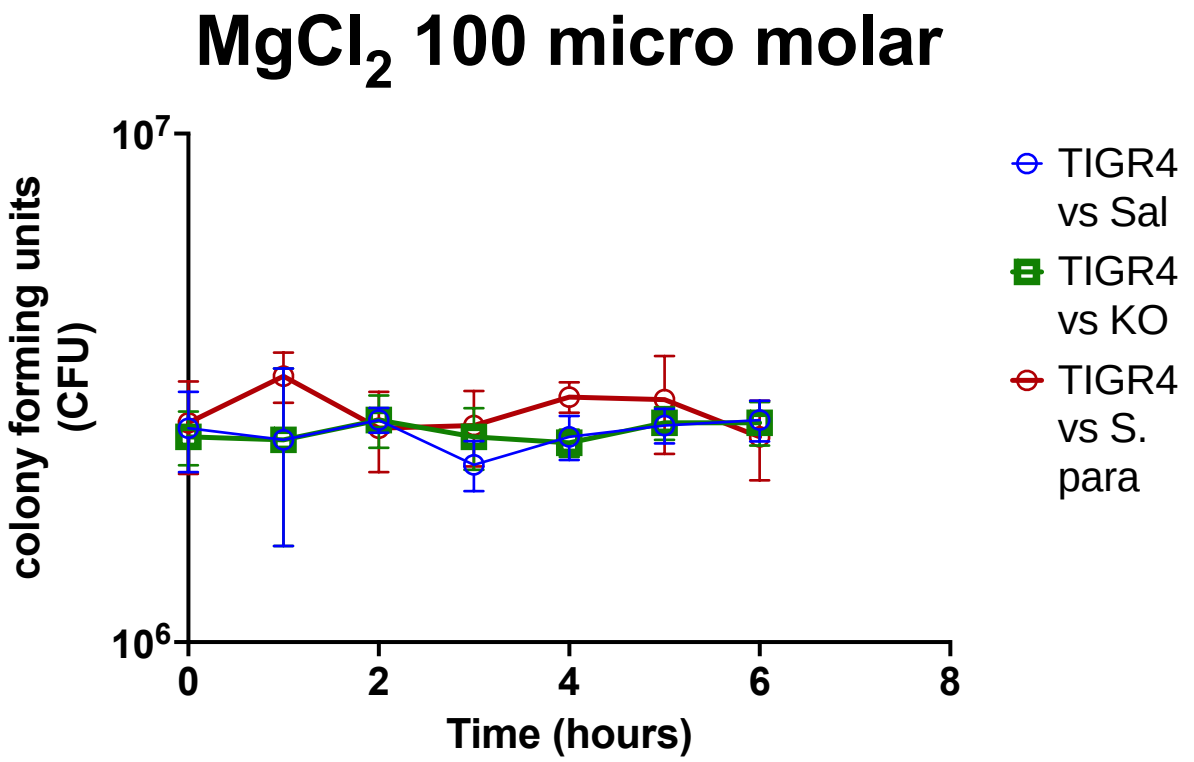

Figure 6-7: Co-culture of TIGR4 and closely related streptococcus species in in metal ion-depleted BHI medium supplemented with $MgCl_2$ 100μM. Red line represents inhibition assay on co-culture of producer TIGR4 WT and the target optochin resistant *S. parasanguinis*. Blue line represents inhibition assay on co-culture of producer TIGR4 WT and the target optochin resistant *S. salivarius*. Values represent the means of CFUs obtained from triplicate cultures plotted against time (hours). Measurements showed less than 1% differences. The error bars are shown in the graphs and readings are mean of three.

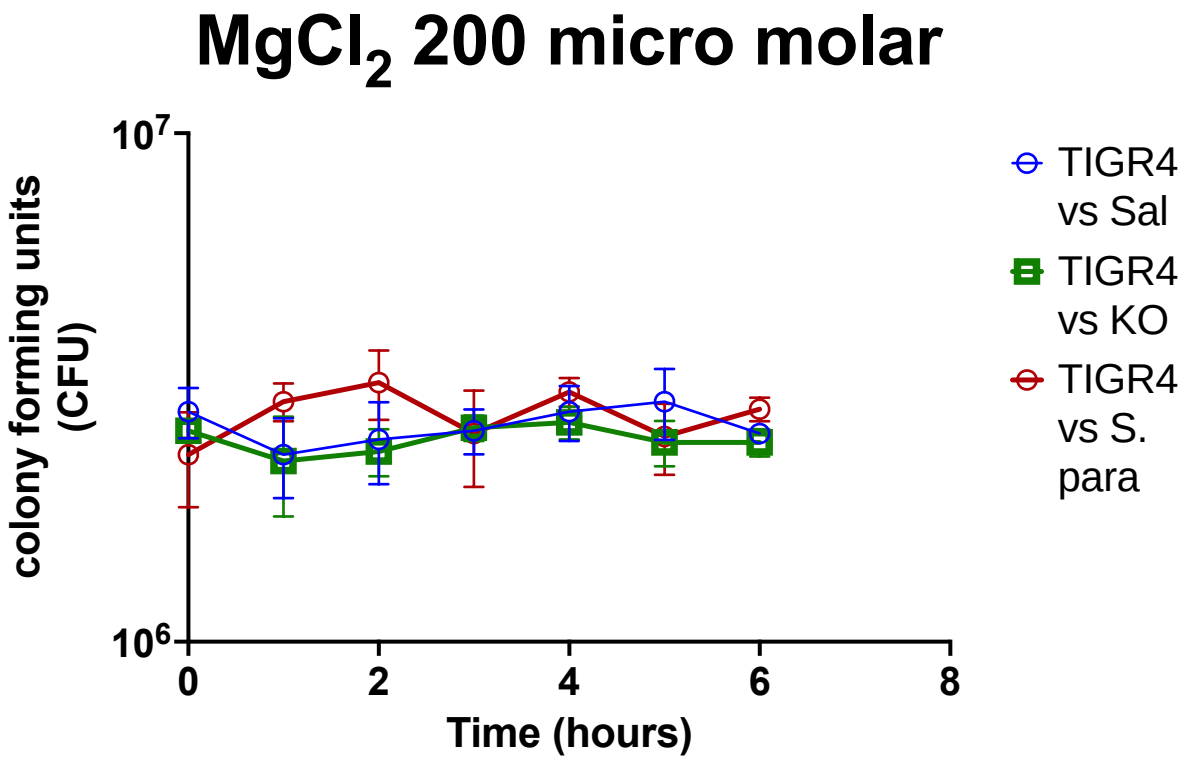

Figure 6-8: Co-culture of TIGR4 and closely related streptococcus species in in metal ion-depleted BHI medium supplemented with $MgCl_2$ 200µM**.** Red line represents inhibition assay on co-culture of producer TIGR4 WT and the target optochin resistant *S. parasanguinis*. Blue line represents inhibition assay on co-culture of producer TIGR4 WT and the target optochin resistant *S. salivarius*. Values represent the means of CFUs obtained from triplicate cultures plotted against time (h). Measurements showed less than 1% differences. The error bars are shown in the graphs and readings are mean of three.

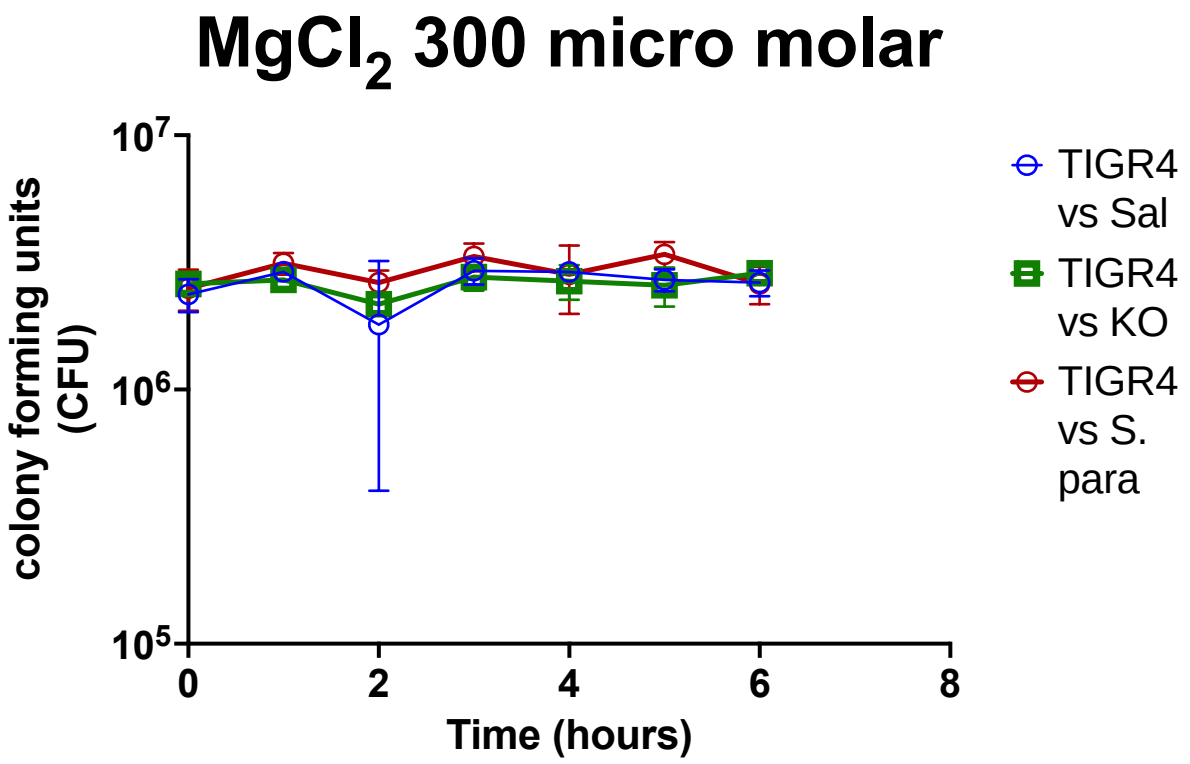

Figure 6-9: Co-culture of TIGR4 and closely related streptococcus species in in metal ion-depleted BHI medium supplemented with $MgCl_2$ 300µM. Red line represents inhibition assay on co-culture of producer TIGR4 WT and the target optochin resistant *S. parasanguinis*. Blue line represents inhibition assay on co-culture of producer TIGR4 WT and the target optochin resistant *S. salivarius*. Values represent the means of CFUs obtained from triplicate cultures plotted against time (h). Measurements showed less than 1% differences. The error bars are shown in the graphs and readings are mean of three.

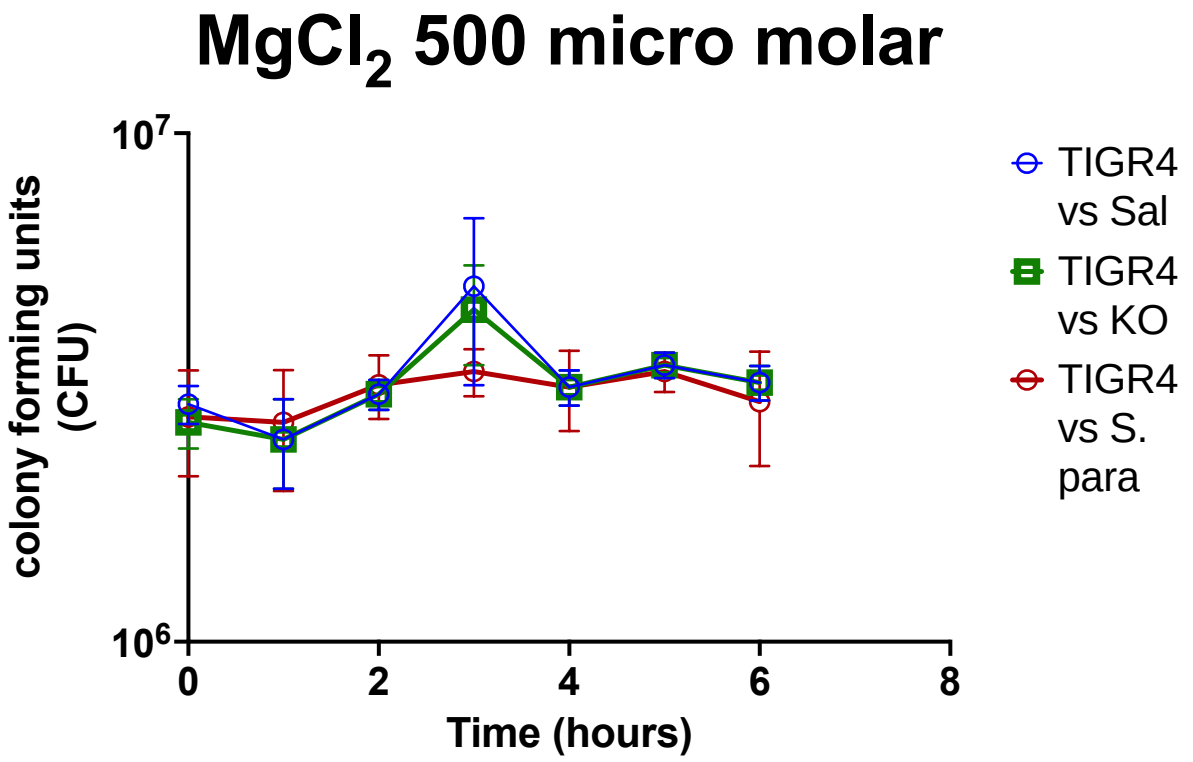

Figure 6-10: Co-culture of TIGR4 and closely related streptococcus species in in metal ion-depleted BHI medium supplemented with $MgCl_2$ 500µM. Red line represents inhibition assay on co-culture of producer TIGR4 WT and the target optochin resistant *S. parasanguinis*. Blue line represents inhibition assay on co-culture of producer TIGR4 WT and the target optochin resistant *S. salivarius*. Values represent the means of CFUs obtained from triplicate cultures plotted against time (h). Measurements showed less than 1% differences. The error bars are shown in the graphs and readings are mean of three.

6.2.5 Co-culture of TIGR4 and closely related Streptococcus species at different Mn^{2+} concentrations

To determine whether the addition of Mn^{2+} influences the inhibition achieved by bacteriocins of the producer TIGR4 WT strain, we cultured the organism in a metal ion-depleted BHI medium supplemented with different concentrations of Mn^{2+} ion. Figure 6.11 shows that the growth curves for co-culture of TIGR4 WT and *S. parasanguinis* in metal ion-depleted BHI medium supplemented with 2µM $MnSO_4$. *S. parasanguinis* maintained the growth at 10^4-10^5 when measured on an optochin agar plate. This data confirms that the TIGR4 WT cannot inhibit *S. parasanguinis* in metal ion-depleted BHI medium supplemented with 2µM $MnSO_4$. This means Mn^{2+} is not a suitable cation to promote the inhibition of *S. parasanguinis* by the WT TIGR4 strain. Similar results were noted when metal ion-depleted BHI medium was

supplemented with higher concentrations Mn^{2+} (5, 10, and 50 μM). However, the growth curve for co-culture of TIGR4 WT and TIGR4 KO was depleted at two hours and persisted up to six hours as shown in Figures 6.12-6.14. This data confirms that TIGR4 WT can inhibit TIGR4 KO in metal ion-depleted BHI medium supplemented with 2, 5, 10, and 50 μM of $MnSO_4$. Values represent the means of triplicate readings of CFUs.

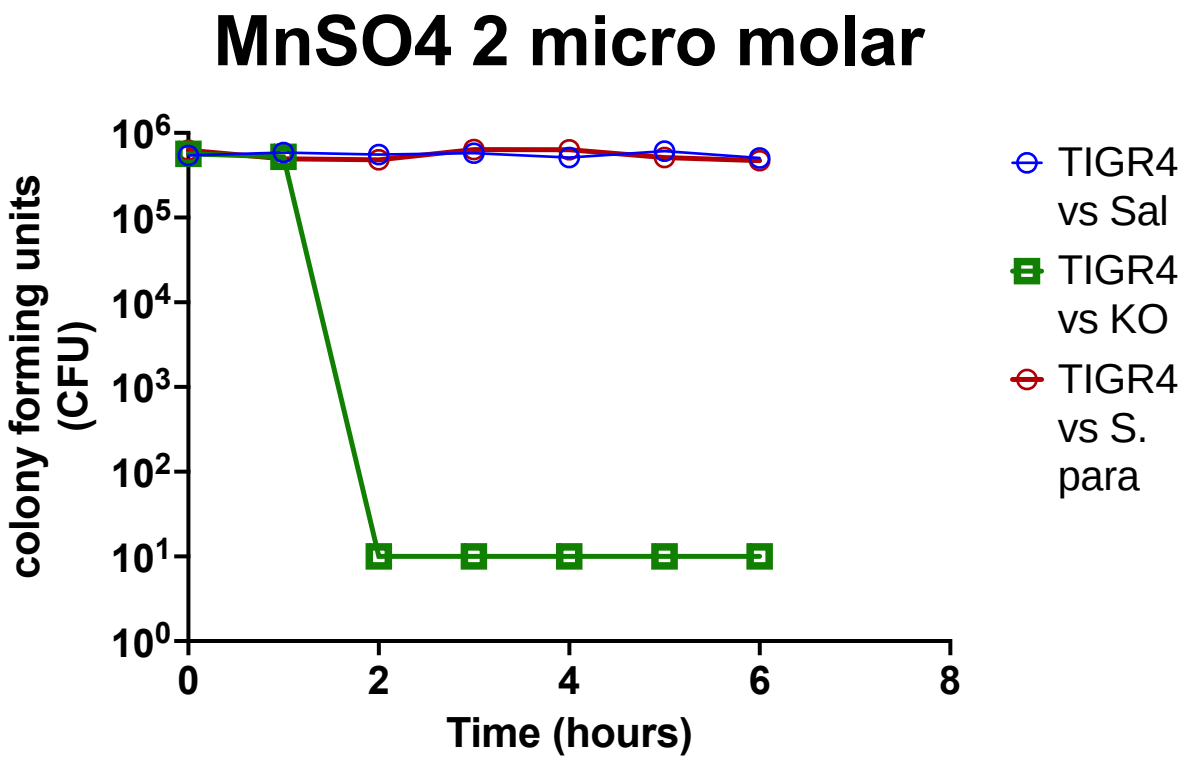

Figure 6-11: Co-culture of TIGR4 and closely related streptococcus species in in metal ion-depleted BHI medium supplemented with $MnSO_4$ 2μM. Red line represents inhibition assay on co-culture of producer TIGR4 WT and the target optochin resistant *S. parasanguinis*. Blue line represents inhibition assay on co-culture of producer TIGR4 WT and the target optochin resistant *S. salivarius*. Values represent the means of CFUs obtained from triplicate cultures plotted against time (h). Measurements showed less than 1% differences. Error bars are shown on the graph but it does not appear because the 3 readings are close to each other which indicates good handing technique.

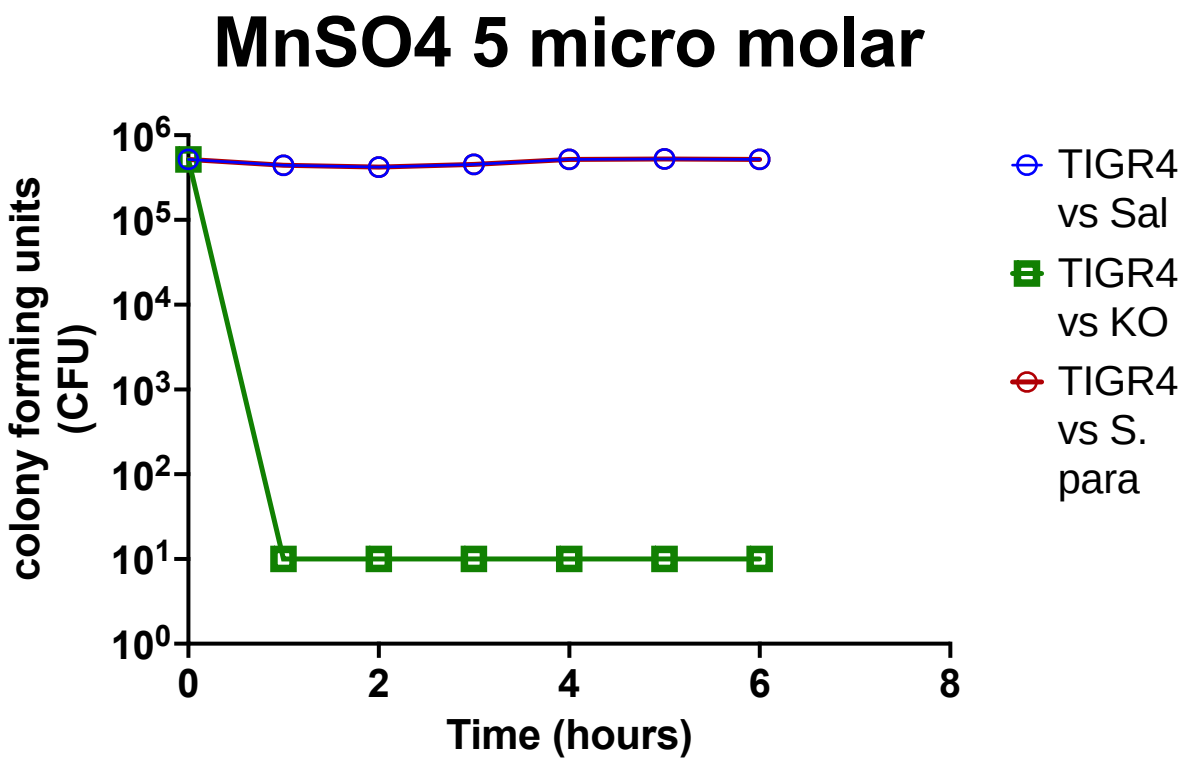

Figure 6-12: Co-culture of TIGR4 and closely related streptococcus species in in metal ion-depleted BHI medium supplemented with $MnSO_4$ 2µM. Red line represents inhibition assay on co-culture of producer TIGR4 WT and the target optochin resistant *S. parasanguinis*. Blue line represents inhibition assay on co-culture of producer TIGR4 WT and the target optochin resistant *S. salivarius*. Values represent the means of CFUs obtained from triplicate cultures plotted against time (h). Measurements showed less than 1% differences. Error bars are shown on the graph but it does not appear because the 3 readings are close to each other which indicates good handing technique.

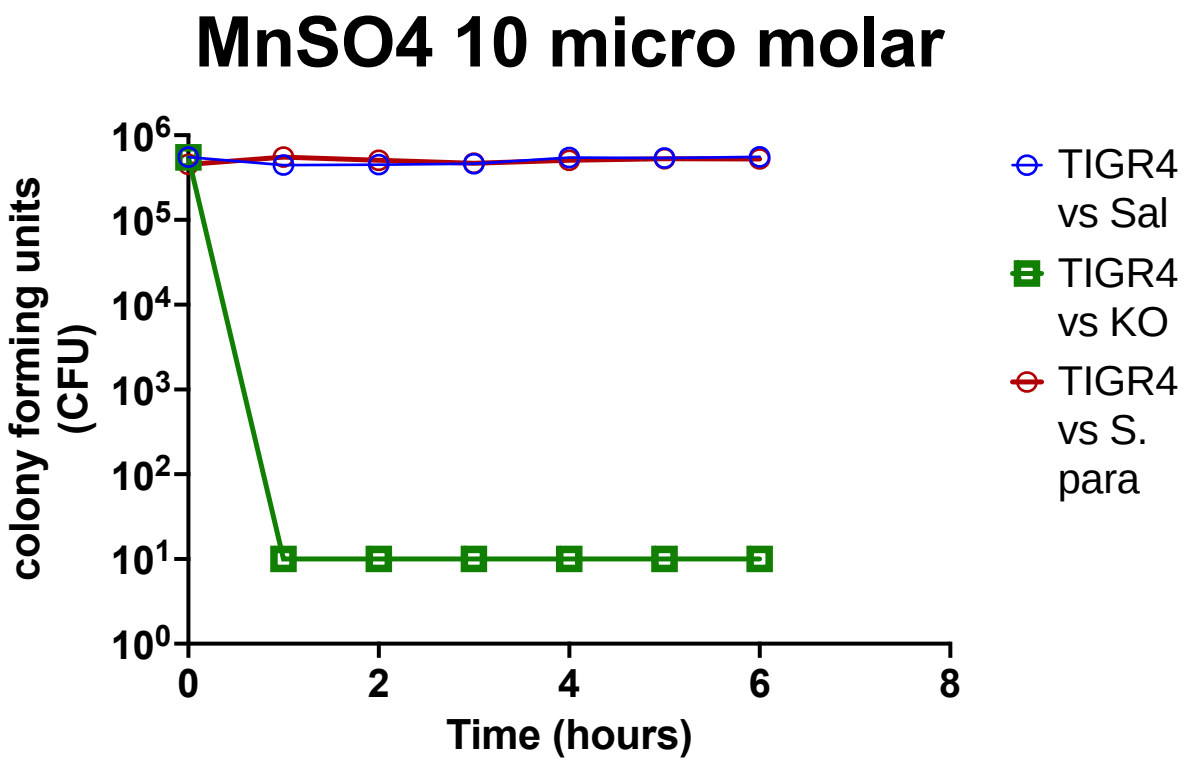

Figure 6-13: Co-culture of TIGR4 and closely related streptococcus species in in metal ion-depleted BHI medium supplemented with $MnSO_4$ 10μM. Red line represents inhibition assay on co-culture of producer TIGR4 WT and the target optochin resistant *S. parasanguinis*. Blue line represents inhibition assay on co-culture of producer TIGR4 WT and the target optochin resistant *S. salivarius*. Values represent the means of CFUs obtained from triplicate cultures plotted against time (h). Measurements showed less than 1% differences. Error bars are shown on the graph but it does not appear because the 3 readings are close to each other which indicates good handing technique.

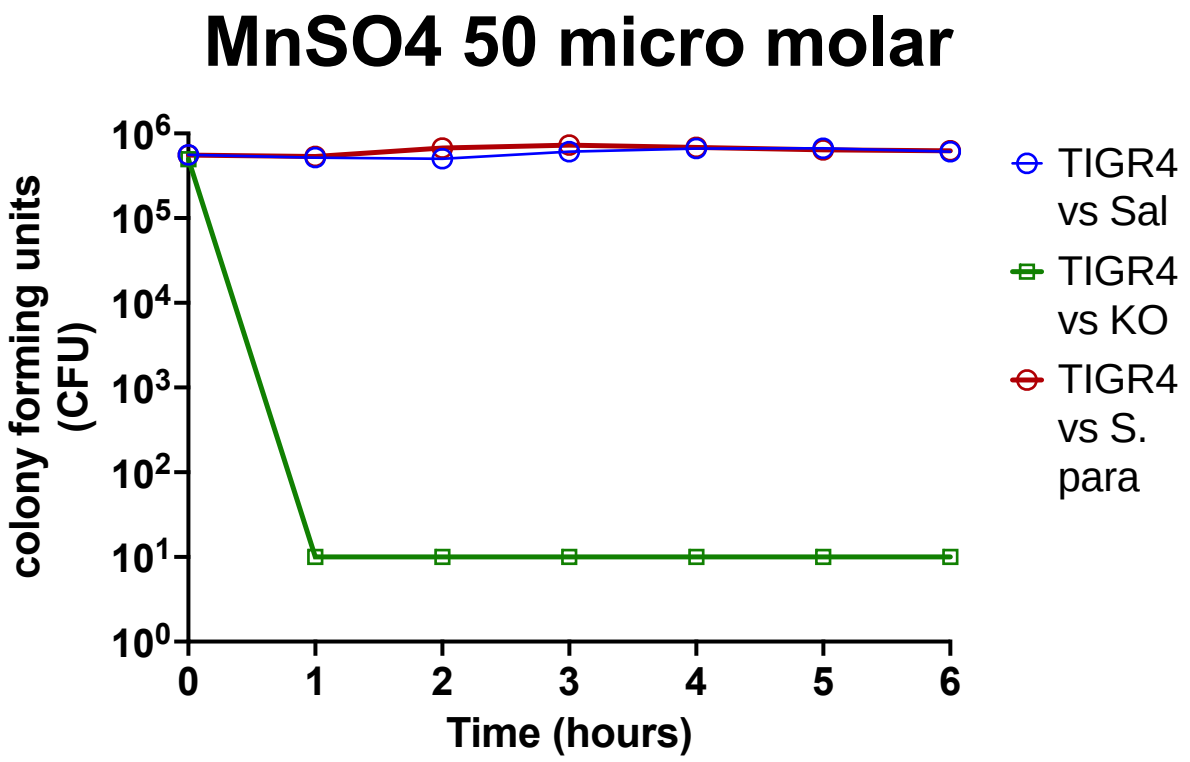

Figure 6-14: Co-culture of TIGR4 and closely related streptococcus species in metal ion-depleted BHI medium supplemented with $MnSO_4$ 50μM. Red line represents inhibition assay on co-culture of producer TIGR4 WT and the target optochin resistant *S. parasanguinis*. Blue line represents inhibition assay on co-culture of producer TIGR4 WT and the target optochin resistant *S. salivarius*. Values represent the means of CFUs obtained from triplicate cultures plotted against time (h). Measurements showed less than 1% differences. Error bars are shown on the graph but it does not appear because the 3 readings are close to each other which indicates good handing technique.

6.2.6 Co-culture of TIGR4 and closely related Streptococcus species at different Fe^{3+} concentrations

To determine whether the addition of Fe^{3+} influenced the inhibition achieved by bacteriocins of the producer TIGR4 WT strain, we cultured the organism in a metal ion-depleted BHI medium supplemented with different concentrations of Fe^{3+} ions. Figure 6.15shows the growth curves for co-culture of TIGR4 WT and *S. parasanguinis* in metal ion-depleted BHI medium supplemented with 2μM $FeCl_3$. *S. parasanguinis* maintained the growth at 10^4-10^5 when measured on an optochin agar plate. This data confirms that the TIGR4 WT cannot kill *S. parasanguinis* in metal ion-depleted BHI medium supplemented with 2μM$FeCl_3$. Similar results were observed when metal ion-depleted BHI medium was supplemented with higher

concentrations of Fe^{3+} (5, 10 and 50 μM). However, the growth curves for co-culture of TIGR4 WT vs *S. salivarius* and TIGR4 vs TIGR4 KO show limitation of the growth of *S. salivarius* and TIGR4 KO at two hours and up to six hours as shown in Figures 6.16-18. This data confirms that inhibition can be restored when 2,5, 10 and 50 μMof $FeCl_3$ is added back to the BHI depleted medium. Values represent the means of triplicate readings of CFUs.

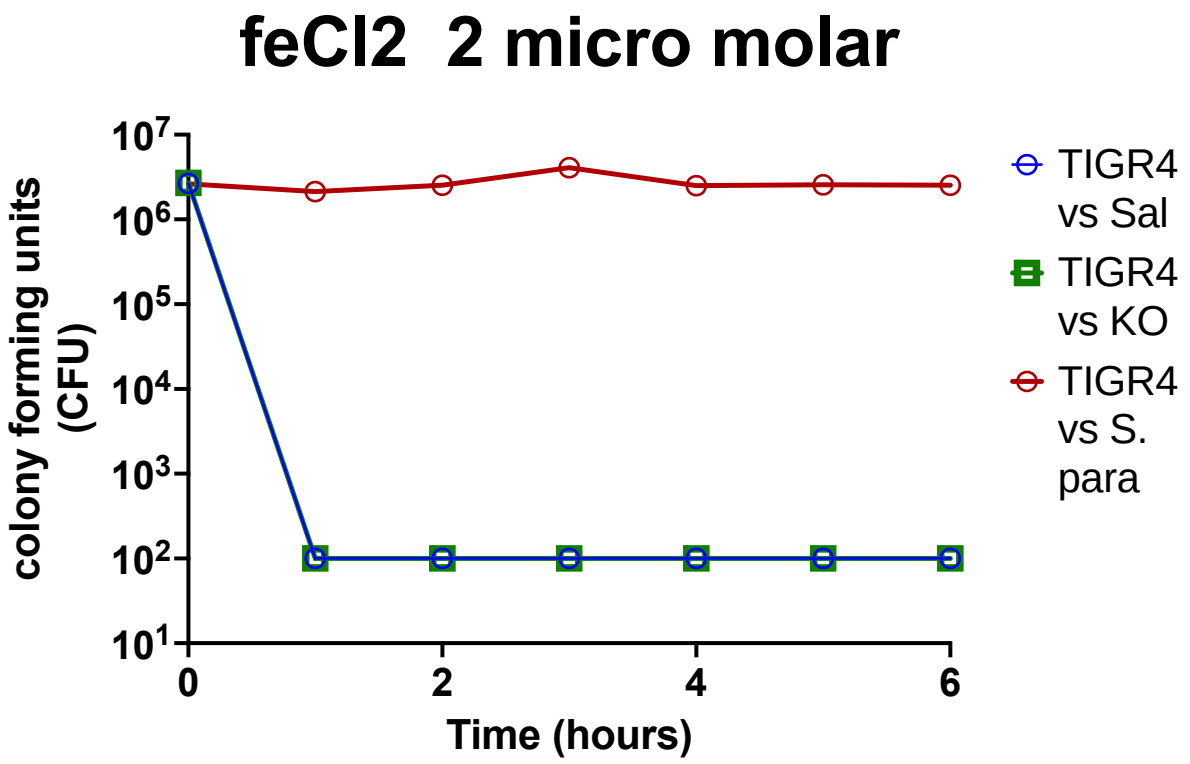

Figure 6-15: Co-culture of TIGR4 and closely related streptococcus species in metal ion-depleted BHI medium supplemented $FeCl_2$ 2μM. Red line represents inhibition assay on co-culture of producer TIGR4 WT and the target optochin resistant *S. parasanguinis*. Blue line represents inhibition assay on co-culture of producer TIGR4 WT and the target optochin resistant *S. salivarius*. Values represent the means of CFUs obtained from triplicate cultures plotted against time (h). Measurements showed less than 1% differences. Error bars are shown on the graph but it does not appear because the 3 readings are close to each other which indicates good handing technique.

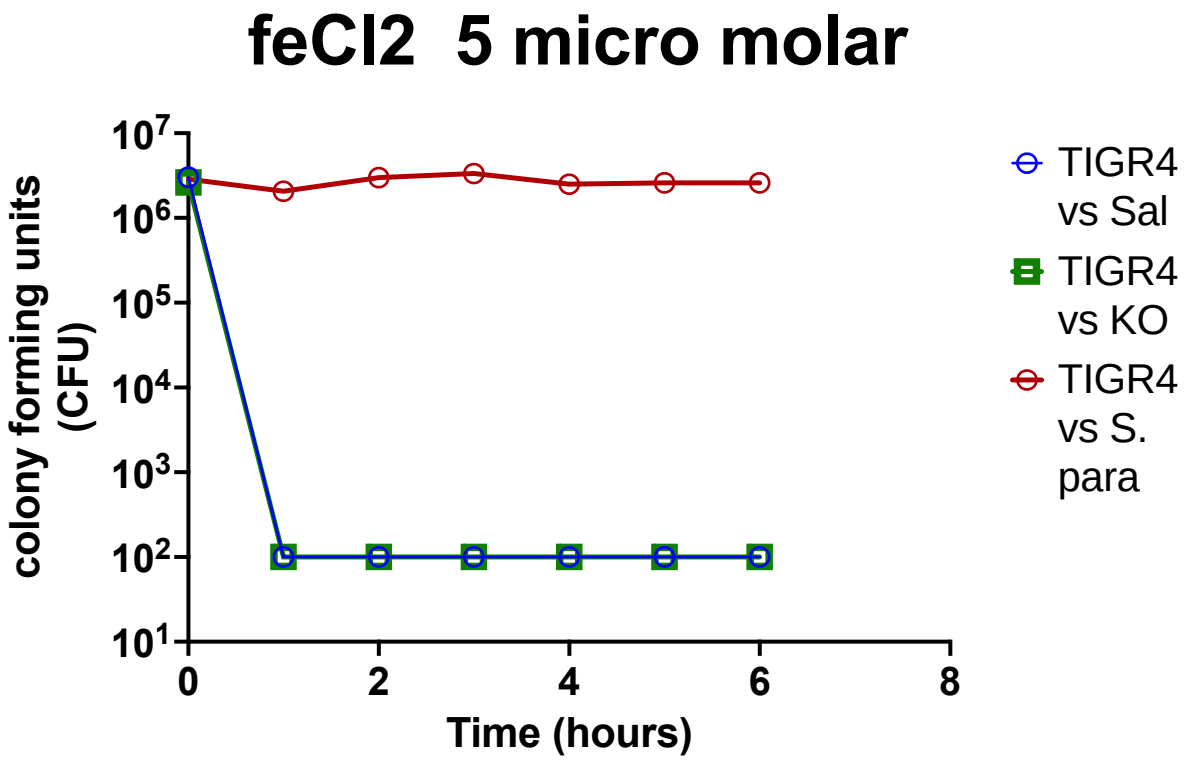

Figure 6-16: Co-culture of TIGR4 and closely related streptococcus species in metal ion-depleted BHI medium supplemented $FeCl_2$ 5μM. Red line represents inhibition assay on co-culture of producer TIGR4 WT and the target optochin resistant *S. parasanguinis*. Blue line represents inhibition assay on co-culture of producer TIGR4 WT and the target optochin resistant *S. salivarius*. Values represent the means of CFUs obtained from triplicate cultures plotted against time (h). Measurements showed less than 1% differences. Error bars are shown on the graph but it does not appear because the 3 readings are close to each other which indicates good handing technique.

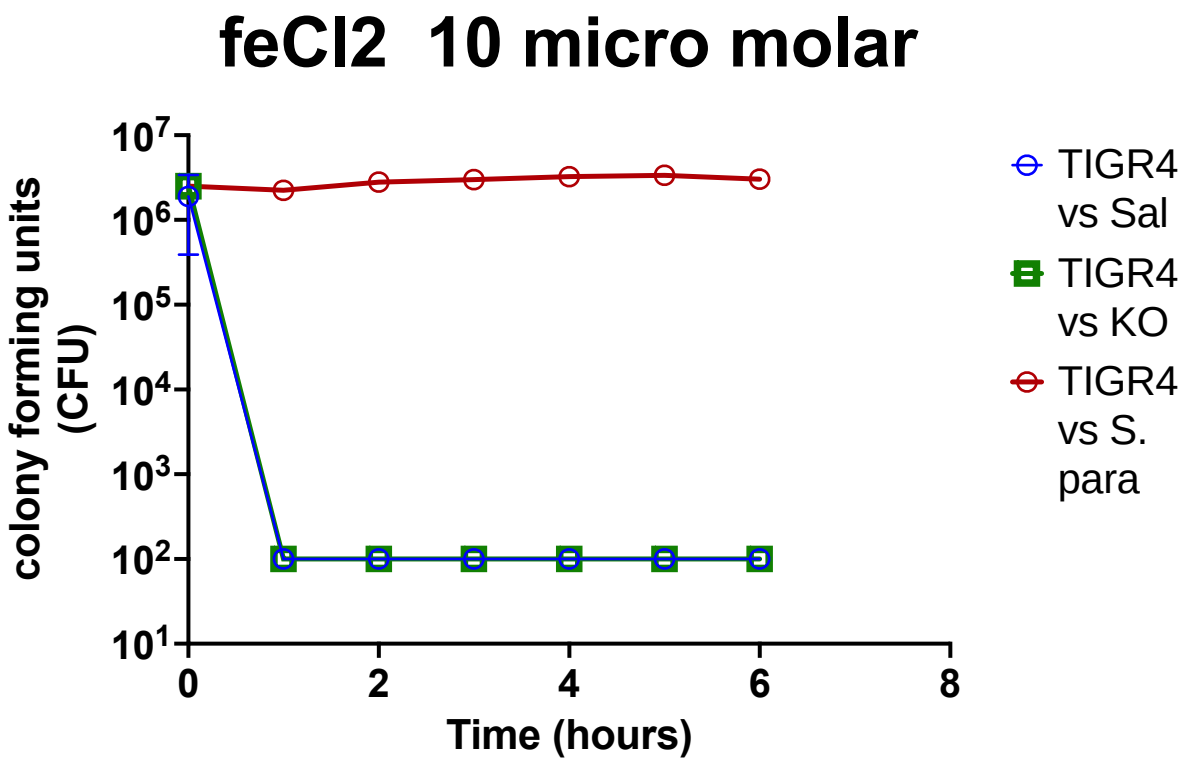

Figure 6-17: Co-culture of TIGR4 and closely related streptococcus species in metal ion-depleted BHI medium supplemented $FeCl_2$ 10μM. Red line represents inhibition assay on co-culture of producer TIGR4 WT and the target optochin resistant *S. parasanguinis*. Blue line represents inhibition assay on co-culture of producer TIGR4 WT and the target optochin resistant *S. salivarius*. Values represent the means of CFUs obtained from triplicate cultures plotted against time (h). Measurements showed less than 1% differences. Error bars are shown on the graph but it does not appear because the 3 readings are close to each other which indicates good handing technique.

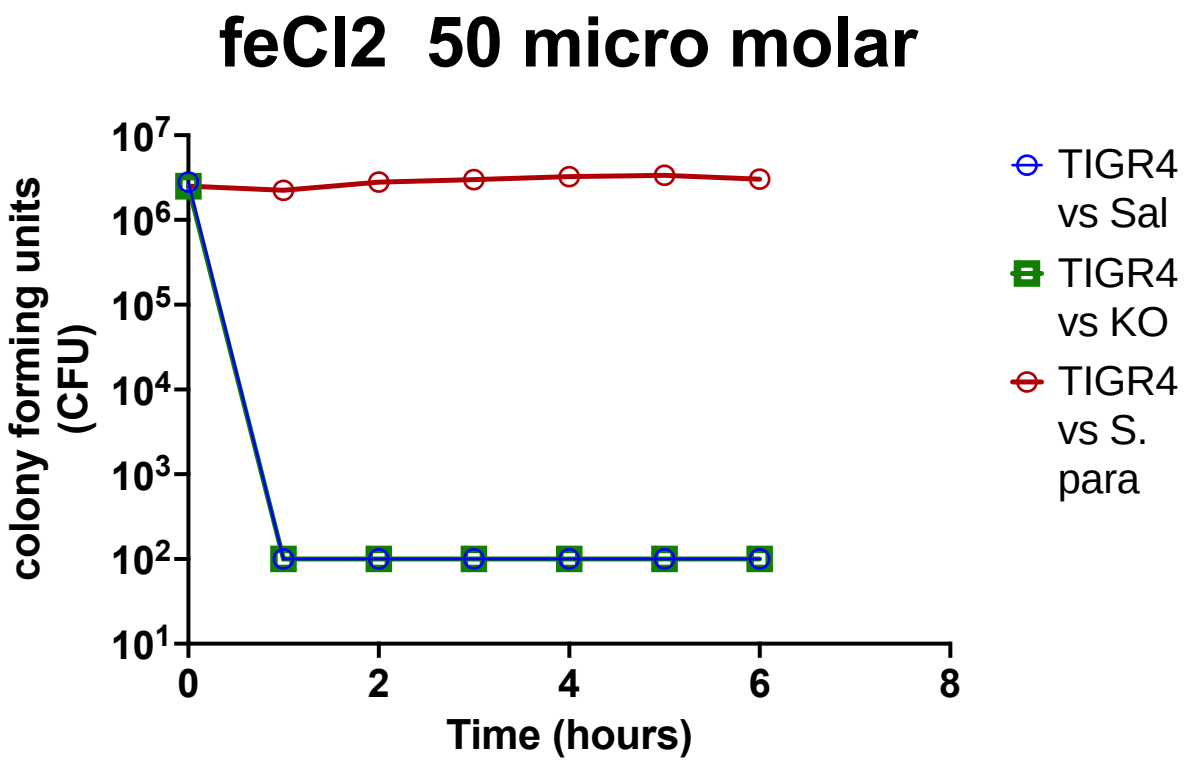

Figure 6-18: Co-culture of TIGR4 and closely related streptococcus species in metal ion-depleted BHI medium supplemented $FeCl_2$ 50µM. Red line represents inhibition assay on co-culture of producer TIGR4 WT and the target optochin resistant *S. parasanguinis*. Blue line represents inhibition assay on co-culture of producer TIGR4 WT and the target optochin resistant *S. salivarius*. Values represent the means of CFUs obtained from triplicate cultures plotted against time (h). Measurements showed less than 1% differences. Error bars are shown on the graph but it does not appear because the 3 readings are close to each other which indicates good handing technique.

6.3 Discussion and Conclusion

The present study evaluated the role of some metal ions in the inhibition efficiency of bacteriocins produced by the TIGR4 WT strain of *S. pneumoniae.* We found that TIGR4 inhibition of *S. parasangunis* was abolished in metal ion deficient media, and that replacement of either iron, manganese, calcium or magnesium could not restore inhibition. In contrast, although inhibition of the TIGR4 Blp KO strain was abolished in metal ion-deficient media, this could be restored by addition of iron or manganese cations, but not calcium or magnesium. Also, although inhibition of *S. salivarius* strain was abolished in metal ion-deficient media, this can be restored by addition of iron, but not manganese, calcium or magnesium cations.

S. pneumoniae have cationic ABC transporter lipoproteins, which are attached to the membrane, where they provide binding sites for the attachment of cationic substrates to be transported. ABC transporters also contain membrane permease(s) for membrane permeation and ATPases for active transmembrane transportation of the metal ions (Durmort *et al.*, 2020). The available concentration of different metal cations in mammalian hosts are tightly controlled and this could, at least in part, explain why cationic ABC transporters have different affinities ($Mn^{2+} > Fe^{2+} > Zn^{2+}$) for the acquisition of these essential metal cations (Durmort et al., 2020). Therefore, *S. pneumoniae* can utilize Fe^{2+} in the absence of Mn^{2+}, and Zn^{2+} in the absence of both Mn^{2+} and Fe^{2+}.

In metal ion-depleted BHI media, Fe^{2+} becomes the second preferred alternative divalent metal ion for maintaining the physiologic processes of *S. pneumoniae*. The intracellular concentration of the Fe^{2+} increased with increasing depletion of Mn in the growth medium. The mechanism of the compensatory effect of Fe^{2+} for Mn in *S. pneumoniae* was demonstrated to be facilitated by 16 types of Fe- or Mn-binding metalloproteins. In Fe^{2+} deficiency, the organism was demonstrated to overexpress ABC transporter lipoproteins, particularly PiaA (SPD_0915), PiuA (SPD_1652), PitA(SPD_0226), SPD_1609, and PsaA (SPD_1463) (Cao et al., 2018). ABC transporter lipoproteins are attached to the membrane, where they provide binding sites for the attachment of substrates to be transported. ABC transporters also contain membrane permease(s) for the formation of pores in the membrane and ATPases for active transmembrane transportation of metal cations (Durmort et al., 2020). The over-expression of ABC lipoproteins in Mn^{2+} deficiency suggested that it is more efficient for Mn^{2+} to be transported by ABC transporters across the outer membrane than it is for Fe^{2+}. Thus, more abundant ABC transporters are required to effectively transport Fe^{2+} across the OM, as a compensatory effect. Bacteriocins exhibit bactericidal effects by increasing plasma membrane fluidity and the formation of ion channels to increase the release of lactate dehydrogenase (LDH). Intra-cellular

depletion of LDH promotes the intra-cellular accumulation of reactive oxygen species (ROS), activates apoptotic pathways (bax/bcl2), and inhibits mitochondrial energy metabolism, leading to the inhibition of the target bacterial cells (Huang *et al.*, 2021). However, to achieve its bactericidal effects, bacteriocins need to bind their target strain, while sparing receptors for the producer strains. As outlined in the Introduction, we hypothesised that metal ion transporters might play a role in bacteriocin targeting and/or transport into cells. Therefore, the present study evaluated the inhibition effect of wildtype (WT) TIGR4 strain towards its rivals, the Blp knockout strain (TIGR4 Blp KO), *S. salivarius* and *S. parasanguinis* in the presence or absence of different concentrations of metal ions.

Our results showed that the inhibition produced by TIGR4 against TIGR4 Blp KO, *S. salivarius* and *S. parasanguinis* was abolished in ion-depleted media. However, the addition of either Fe^{3+}or Mn^{2+} could restore the bactericidal efficiency of bacteriocin produced by the TIGR4 WT strain towards TIGR4 Blp KO and *S. salivarius* but not *S. parasanguinis*. Calcium and magnesium ions had no effect on either target. Fe^{3+} was used instead of Fe^{2+} because its more oxidative stable. Thus, our original hypothesis that depleting metal ions might up regulate a transporter required for bacteriocin action leading to greater inhibition was not substantiated.

The lack of inhibition in ion-depleted media could be due to a lack of production of bacteriocins and/or a failure of target inhibition. Addition of metal ions back to the depleted media allowed some further conclusions to be made. With the addition of Fe^{3+} or Mn^{2+}, inhibition of TIGR4 Blp KO was restored, but not of *S. parasanguinis.* The addition of Fe^{3+} also restore the killing of *S. salivarius* but not Mn^{2+}. Thus, lack of inhibition of this organism was not due to failure of production under these conditions but must reflect a requirement for these ions in the inhibition mechanism. The restoration of inhibition of TIGR4 Blp KO by either Fe^{3+} or Mn^{2+}, and *S. salivarius* by the addition of Fe^{3+}, could be due to bactericin binds to accessory receptors of these metal ions or production or a direct requirement for these ions in the bacteriocin

induced cell inhibition. Many bacteriocins have been found to gain entry into cells using metal cations. The ABC transporter and its accessory proteins could be useful in aiding the exportation of bacteriocins into the target cells (Sidhu and Nehra, 2021). The cationic ABC transporter lipoproteins provide anionic binding sites for the attachment of metal cations. This, therefore, suggests that for bacteriocins to be transported, they must exhibit cationic behaviour. Indeed, for instance, LAB bacteriocins with 20–60 amino acid residues are generally cationic and hydrophobic or amphiphilic molecules, which exhibit membrane-permeabilizing ability (Hernández-González *et al.*, 2021). Therefore, the cationic bacteriocins bind cationic ABC transporter lipoproteins, where they access their primary receptor (anionic lipids) in the cytoplasmic membrane as the initial step for pore formation. Cationic ABC transporters could promote pore formation via their membrane permeases to aid the entry of bacteriocins (Durmort et al., 2020). Interestingly, unlike antibiotics, bacteriocins exert their bactericidal effects via a unique mechanism of action, which involves binding the pyrophosphate moiety of the anionic lipids (lipid-II), therefore, unlikely to develop bacterial resistance (Huang et al., 2021). Findings from the present study and evidence from the literature suggest that bacteriocins are not transported as metal cations, but are transported by accessory binding proteins in the ABC transporters during transport of $Fe^{3+/2+}$ or Mn^{2+} (Hernández-González et al., 2021).

The present study also evaluated whether the TIGR4 WT strain kills the susceptible strains TIGR4 Blp KO, *S. salivarius* and *S. parasanguinis* in metal ion-depleted BHI medium and whether supplementation with another cation including Ca^{2+} and Mg^{2+}, offered a similar compensatory effect as Fe^{3+}. Co-cultures of TIGR4 WT and TIGR4 Blp KO or*S. salivarius*or *S. parasanguinis* in metal ion-depleted BHI medium supplemented with Ca^{2+} and Mg^{2+} did not kill the rival strains. This indicated that the two divalent cations do not support the exportation of bacteriocins produced by TIGR4 WT across the OM of the rival strains to induce bactericidal

effects. This finding is consistent with the literature where it is demonstrated that Fe^{2+} and Mn^{2+} enhance while $Cu^{2+,}$ K^{+}, Ca^{2+}, Zn^{2+}, $Mg^{2+,}$ and NaCl inhibit the bactericidal effects of bacteriocins secreted by LAB (Matevosyan et al., 2019; Zhang et al., 2018).

The current study indicates that Ca^{2+} concentration plays a role in the growth of pneumococcus species. In this study, we have been able to determine the levels of concentrations that are optimal for *S. pneumoniae* growth in concentrations of 100μM, 200μM, 500μMand 1000 μM for calcium ion. There are limited studies that have attempted to compare these range of concentrations on *S. pneumoniae* strains of TIGR4 co-cultured with *S. parasanguinis.* Our study agrees with Brown et al., (2017) study on the increased concentration of Zinc in growth media for *S. pneumoniae* strain growth. According to Brown et al., (2017), inhibition of bacteria growth at high concentrations of zinc may be resulting from the inability of MgtA transporter to accommodate high level concentrations of metal ions. The presence of other ions in the media will overwhelm the functions of the transporter MgtA, which transports magnesium ions and exports Ca^{2+} ions. Therefore, under low concentration strain TIGR4 leads to optimal growth, but the concentration turns toxic at higher concentrations (Neef et al., 2011).

Colonization and infection of *S. pneumoniae* have been widely studied. The major concentration of the studies lies in molecular factors determining bacterial species' invasion and pathogenesis (Weiser, Ferreira, & Paton, 2018). Additionally, other studies concentrated on the effect of metal in the downregulation or upregulation of the pathogenic organism. Besides Iron (Fe^{2+}), the widely studied metals in determining the upregulation or downregulation of *S. pneumoniae* are Zinc (Zn^{2+}) and Manganese (Mn^{2+}). These metals are essential cofactors for various enzymatic activities and mediation of oxidative stress resistance (Weiser, Ferreira, & Paton, 2018). On the other hand, studies on gene and lipoproteins expression have been undertaken to determine the types of genes and virulent lipoproteins and their effect in expressing virulent factors and the host's immunological response. Therefore, it

implies that understanding the effect of these transition metals and the involved genes will be the basis for understanding Pneumococcal colonization and infection control approaches.

Metal ions play an important role in life forms through interaction with proteins to facilitate activities of the various forms of life. Manganese is a cofactor in superoxide dismutase (SOD), prominently protecting organisms against oxidative stress. The Mn^{2+}plays a protection role by catalysing the dis-mutation of superoxide to produce hydrogen peroxide and oxygen. The manganese cation is involved in resistance to oxidative stress by directly detoxifying reactive oxygen species (ROS), signalling of regulation of genes that protect bacteria against ROS, and playing an indirect role as a cofactor for enzymes (Yesilkaya *et al.*, 2013). Thus, Mn^{2+} plays an important role in Streptococci physiology and virulence.

In a study conducted in the university of Mississippi laboratory, using a combination of transcriptomic approaches, it was found that the Mn^{2+} regulated expression of *pcp*A and *prt*A genes responsible for pneumococcal virulence. Additionally, the study team found that Mn^{2+}-regulated enzymes (SPxB and Mn^{2+} SodA) are involved in pneumococcal responses to oxidative stress (Ogunniyi *et al.*, 2010). The study indicates that the presence of Mn^{2+} in the pneumococcal extracellular environment promotes colonization and infection of *S. pneumoniae*, causing diseases. However, the presence and activity of Mn^{2+} depend on the presence of other enzymatic metal cofactors, such as Zinc (Zn^{2+}). Where the amount of $Zn^{2+}$is insignificant in the extracellular environment, Mn^{2+} uptake by the *S. pneumoniae* increases sizeably and promotes the expression of virulence expression genes on the bacteria cell surfaces and the body's immunological response (Ogunniyi *et al.*, 2010).

The uptake of Mn^{2+} through the bacterial transporters into its endo-cellular environment plays a role in gene expression. According to Ogunniyi *et al.*, (2010) grown *psaA* mutants investigation in the absence of Mn^{2+} slowed the growth of the *S. pneumoniae*. At the same time, the presence of Mn^{2+} had a growth rate comparable to the wild-type. Thus, it implies that the

presence of Mn^{2+} would increase the expression of *psaA*, an important protein that increases resistance against oxidative stress. Further, the bioavailability of Mn^{2+} positively correlates with increased capsular production (McFarland *et al.*, 2021). Considering that the bacterial capsule is an important virulence factor of pathogenic microorganisms, the presence of Mn^{2+} will undoubtedly increase *S. pneumoniae* colonization and infectivity.

Zinc becomes a metal ion of interest in this study because it is often studied together with calcium. Both metals have shown an essential microbial micronutrient involved in homeostasis and lipoprotein expression. *S. pneumoniae* expresses zinc-binding lipoproteins, AdcA and AdcAII, transporters to enhance the ZN efflux system. Brown *et al.* (2017) in vitro study indicated that *S. pneumoniae* encountered with high Zn^{2+}concentrations could form biofilms and larger aggregates. Zinc protects pneumococci against oxidative stress; if present, *S. pneumoniae* forms biofilms, and colonization is enhanced. It is important to note that a high concentration of Zn^{2+}over Mn^{2+} inhibits Mn^{2+}uptake, and the *S. pneumoniae* forms biofilms and colonizes the host (Ogunniyi *et al.*, 2010). Thus, the downregulation of Zn^{2+}concentration is critical in reducing pneumococci colonization and infection.

Metal ions concentration varies from microbial host to host and from one organ or tissue to another. For example, *S. pneumoniae* is likely to experience high variation in Zinc concentrations as they move from the respiratory tract to the lungs and the blood or cerebrospinal fluid. Consequently, the concentrations will affect the bacterial ability to colonize and cause infection. Like other transition metals like iron and manganese, zinc ions are required as components of bacterial protein structures, cofactors, and supporting cellular defences and metabolism. Therefore, the high the zinc concentration in the *S. pneumoniae* extracellular environment, the more the metal ions are transported into the bacteria through zinc transport proteins. The abundance of zinc availability promotes biofilm formation, increases colonization, and interrupts bacterial autolysis.

Previous studies on the zinc concentration effect on the *S. pneumoniae* have shown that zinc availability increase or decreases the colonization of the bacteria. In the presence of high zinc concentrations, *S. pneumoniae* increases cell-to-cell interaction and subsequently forms biofilms and larger aggregates of colony-forming units (Brown*et al.,* 2017). Brown and colleagues observed a 3D plague-like structure in 500 µM zinc concentration cultured *S. pneumoniae* cells. Thus, as zinc concentration increases, biofilms, and cellular aggregation increase. The biofilms protect the bacteria against treatment antibiotics and the host's cellular defences. Thus, biofilms serve as an essential characteristic of pneumococcal colonization and infection since they shield the bacteria against extracellular environmental stressors to increase survival chances.

High zinc concentrations are a negative regulator of autolysin. An autolysin is an enzyme that disintegrates bacterial cells to release DNA. LytA, a major *S. pneumoniae* autolysin, was found to be downregulated with a high zinc concentration (Mellroth et al., 2012). Further, the pneumococci gain protection against autolysin with enhanced zinc availability. The implication is that more biofilms form, and bacterial aggregation survival increases due to enhanced defences against environmental stressors (Brown*et al.,* 2017). In summary, high concentrations of zinc ions enhance cellular aggregation and biofilm formations, increasing the chances of colonization and survival because of enhanced defences against bacterial stressors.

Signal transduction process allow bacteria to sense their environment. The basic mechanism for signal transduction is activation of membrane-bound sensor known as histidine kinase, which is an auto-phosphorylation residue in the cell's intracellular catalytic domain (UlijasZ et al., 2004). This process has been shown to allow bacteria to sense their environment and respond to various environmental cues such as virulence and nutrition. The genome of *S. pneumoniae* allows for signal transduction in a wide range of responses to the environment. The dominating environment for *S. pneumoniae* is a BHI media responsible for growth. Metal

ions serve as nutrients in BHI media that aid this bacterial growth (UlijasZ et al., 2004). Iron serves as a major metal ion and an essential element aiding in growth. The presence of Fe^{2+} in the growth media for TIGR4 increase its concentration in the mucosal surfaces. The ability of the TIGR4 to sequester Fe^{2+} in any concentration determines its growth rate. The TIGR4 growth has been found to occur in low Fe^{2+} concentrations in the media. It is expected that an increase in Fe^{2+} would slow the growth rate (UlijasZ et al., 2004). The TIGR4 has an elaborate mechanism for utilizing ions without the risk of toxicity. The TIGR4 KO genes however raises the question of whether it uses the same mechanism to regulate Fe^{2+} intake. The inhibition essay needs Fe^{2+} concentrations in different levels to determine susceptibility of TIGR4 vs TIGR4 KO. The inhibition assay and experimental susceptibility suggest the genetic mediated iron uptake as responsible for bacteria growth.

In research involving a mouse model to investigate the role of Glutathione, it was found that Glutathione, a lipoprotein expressed by *S. pneumoniae,* plays a role in defending the bacteria against oxidative stress and toxic metal ions concentrations (Potter, Trappetti & Paton, 2012). Glutathione is a protein that the pathogenic organism imports from the extracellular environments through *glutathione S-transferases (GSTs)*, a binding protein of ABC transporter. The proteins play an important role in redox chemistry and oxidative stress tolerance in different organisms' cells. According to the study conducted by Potter, Trappetti & Paton (2012), mutation of the *GST* and glutathione reductase (gor) encoding gene increased resistance of the pneumococci against oxidative stress. The mutation of gor encoding genes in the undertaken study demonstrated the complete dysfunctionality of glutathione reductase activities.

Pneumococci do not express catalase enzymes but code for other putative peroxidases, such as glutathione peroxidases and thiol peroxidase, to detoxify the bacteria. Principally, the *S. pneumoniae* coding for glutathione is to protect the bacteria against toxic concentrations of

metal cations such as Manganese (Mn^{2+}), Zinc (Zn^{2+}), Copper (Cu^{2+}), and Iron (Fe^{2+}) (Potter, Trappetti & Paton, 2012). Depending on the concentration levels of the metal ions in the extracellular environment of the bacteria, the expression of virulence factors will increase to cause colonization and infection or reduce cause inhibition of the pathogens (Potter, Trappetti & Paton, 2012). Therefore, in efforts to establish other treatments and control modes of *S. pneumonia*, paying attention to possible ways of inhibiting the uptake of Glutathione will be essential. If the Glutathione is inhibited, pneumococcal growth will be inhibited, too, whether Zn^{2+}, Cu^{2+}, or $Mn^{2+}$is present. Therefore, further studies on Glutathione's inhibition mechanism are worthwhile because of its potential to inhibit pneumococcal growth.

Iron is important in the cell's physiological functions. *S. pneumoniae* bacteria use haemoglobin (Hb) and haem as the only iron sources from their human host (Olivares-Trejo et al., 2022). The levels of iron required by the bacteria in cellular activities are higher than those found in human tissues and cells. *S. pneumoniae* has evolved to develop several mechanisms of obtaining iron from the host through its iron-binding protein membranes. The bacteria and functions secrete glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to bind the two-iron source, the haem, and the haemoglobin. GAPDH promotes the attack of the pathogen on the tissue cells of the host for successful infections (Olivares-Trejo et al., 2022)**.** *S. pneumoniae* haem-binding protein 37 (Spbhp-37) is the chief membrane involved in acquiring proteins from host cells. Spbhp-37 ensures that the bacteria are viable in the presence of iron sources during its attack on human tissues.

Despite the bacteria lacking ferritin, it has developed coping mechanisms to obtain iron from ferritin sources in the human body. The PspA protein of the pathogen can bind the lactoferrin to the membrane surfaces of the bacteria, especially in tissue with lactoferrin as the only source of iron (Olivares-Trejo et al., 2022). The PiaA hemoglobin-bounding protein is involved in transporting the iron required to the *S. pneumoniae* surface, making it available for the

pathogen's survival. Amino acids components of the Sphbp-37 protein membrane of the pathogen have been linked to the bounding affinity of iron (Olivares-Trejo et al., 2022). Amino acids such as alanine, and alanine are directly bound to the haem and hemoglobin in the host tissues. When the two amino acids are substituted, the bounding of iron sources to the protein membranes is impaired.

Understating the types of transporters and their significance in *S. pneumoniae* colonization and infection is a primary basis for preventing the colonization of the pathogen. The presence of AdcA and AdcAII transporter proteins enhances the virulence of the pneumococci and normalizes the bacterial morphology through the support of Zinc uptake. According to Bayle *et al.* (2011), deletion of the AdcA and AdcAII transporters lowers Zn^{2+}concentration and subsequently lowers colonization of the pneumococci. Zinc is a cofactor that promotes the expression of virulence factors on the bacterial cell surface and the genetic expression of defence factors. Therefore, capitalizing on deleting the metal ion transporters can reduce *S. pneumoniae* colonization and infection.

In conclusion, we have shown that Mn^{2+} and Fe^{3+} are essential for the bacteriocin-mediated inhibition of TIGR4 blp KO by WT TIGR4. Also, Fe^{3+} is essential for the bacteriocin-mediated inhibition of *S. salivarius* by WT TIGR4. Further experiments will be required to differentiate between a requirement of these ions for bacteriocin production, or that they are essential for bacteriocin action. We speculate that these metal cations may play a role in stimulating uptake/attachment of bacteriocins to the target cell.

CHAPTER 7: General Discussion and Conclusion

7.1 Summary of Findings

S. pneumoniae, also known as pneumococcus, is a bacterial species that can colonize and infect the respiratory tract of humans, leading to a wide range of diseases such as pneumonia, sepsis, meningitis, and ear infections. The ability of *S. pneumoniae* to colonize and infect is influenced by several factors. One of the major factors that contribute to the virulence of *S. pneumoniae* is the presence of virulence factors such as capsule, pili, and enzymes. These molecules enable the bacterium to adhere to host cells and evade the host's immune response, which increases the ability of the bacteria to colonize and infect the host. Another important factor is the susceptibility of the host, which can be influenced by several factors such as a weakened immune system, chronic diseases, and certain genetic conditions. People with these conditions are more likely to develop severe infections when they are infected with *S. pneumoniae*. *S. pneumoniae* is primarily transmitted through respiratory droplets, either by direct contact with an infected person or by contact with contaminated surfaces. This makes it easy for the bacteria to spread within households, communities and health care facilities. The overuse or misuse of antibiotics has led to the emergence of antibiotic-resistant strains of *S. pneumoniae*. These strains are able to survive exposure to antibiotics, which makes it difficult to treat pneumococcal infections, especially in patients with severe or recurrent infections. Additionally, living conditions that promote the spread of the bacteria such as crowded living conditions, lack of access to clean water and sanitation can also contribute to the ability of *S. pneumoniae* to colonize and infect. It's important to note that there are different types of *S. pneumoniae* serotypes, and some serotypes are more virulent than others, some are more susceptible to antibiotics, etc. The epidemiology of the disease in a given population may depend on the dominant serotypes circulating. Therefore, understanding the serotype

distribution in a specific population is essential for developing appropriate prevention and treatment strategies.

There is a large number (>100 species) of bacteria belonging to the Streptococcus genus and most of them typically colonize mucous membranes of both humans and animals. Some of the pathogenic Streptococci include *S. pneumoniae, S. pyogenes, S. agalactiae, S. mutans, S. suis,* among which *S. pneumoniae* (Pneumococcus) is the predominant. Pneumococcus has remained of high public health concerns worldwide as it is responsible for the highest number of pneumonia cases globally (Krzyściak *et al.,* 2013). Pneumococcus exhibit significant genetic flexibility and is amenable to natural genetic transformations to acquire new survival phenotypic features (Krzyściak et al., 2013). The present study was prompted by the peculiarity of Pneumococcus in its predominance colonization and infection in human hosts over the rest of the pathogenic Streptococcus species. Co-colonizing pneumococci compete with each other through a diverse array of bacteriocins (pneumocins) and related antimicrobial peptides (Weiser *et al.,* 2018). Therefore, the present study evaluated the molecular factors that give streptococcus the advantage to colonise and outcompete other closely related rival strains and species.

S. pneumoniae's capacity to colonize and infect is regulated by a number of molecular variables. There are several *S. pneumoniae* serotypes, and some serotypes are more virulent than others and may have some of these molecular factors developed than others. This is crucial to keep in mind. The presence of a polysaccharide capsule is one of the key elements that boosts *S. pneumoniae*'s pathogenicity. The ability of the bacterium to elude the host's immunological response and resist phagocytosis is made possible by this capsule, which also boosts the bacterium's capacity to colonize and infect the host. Fimbriae, also known as pili, are a crucial virulence component. By attaching to certain receptors on the surface of the host cell, these hair-like projections aid in *S. pneumoniae*'s ability to cling to the cell. As a result, the bacteria

may cling to and colonize host tissue. In addition, *S. pneumoniae* generates a number of enzymes, including hyaluronidase and neuraminidase, that can break down parts of the host extracellular matrix. These enzymes may facilitate the spread of the bacteria from the respiratory system to other body regions of the host and the infection of other organs.

The capacity of *S. pneumoniae* to get iron from sources produced from the host is a vital component that boosts its pathogenicity. Because of this, the bacteria can live in conditions where it would be impossible for competing strains to get enough iron to thrive. Another method by which *S. pneumoniae* detects and reacts to variations in population density is quorum sensing. By coordinating the production of virulence factors, the bacteria are able to adapt to various conditions. By being resistant to medications, *S. pneumoniae* can potentially outcompete other strains or species. As a result, it may live and develop when medical interventions might otherwise destroy other germs. Furthermore, *S. pneumoniae* can create a number of chemicals that can hinder the host's immunological response and aid the germs in evading it. Phosphorylcholine and pneumolysin are two examples of these compounds. As a result, *S. pneumoniae* possesses various molecular characteristics that help it colonize and outcompete other closely related competitor strains and species. Understanding these characteristics is critical for creating successful pneumococcal infection prevention and treatment techniques.

The present study aimed to investigate the role of the blp locus in bacteriocin production in *S. pneumoniae* TIGR4, a virulent serotype 4 strain that is a frequent clinical isolate in patient blood samples. bacteriocin production in *S. pneumoniae* is encoded by genes located at the blp locus, and previous studies have suggested that the growth of TIGR4 may be inhibited by certain strains of *S. pneumoniae*. In order to determine the role of the blp locus in TIGR4, the entire locus was deleted using a Sweet Janus (SJ) cassette construct. The resulting TIGR4 Blp knockout strain was then co-cultured with both TIGR4 wild type and *S. parasanguinis*. The

results of this co-culture revealed that TIGR4 actually produces bacteriocin, as the TIGR4 Blp knockout strain was inhibited by TIGR4 wild type, but not by *S. parasanguinis* or other closely-related strains. Furthermore, the TIGR4 wild type strain was found to produce BlpC, a bacteriocin, but the TIGR4 Blp knockout strain did not. These findings refute the hypothesis that TIGR4 does not produce bacteriocins at all and clarify previous findings by Dawid et al. (2007) that TIGR4 may be inhibited by certain strains of *S. pneumoniae*. It should also be noted that TIGR4 along with other strains such as serotype 6A, WU2, serotype 2 strain D39, serotype 6A strain 6A10, and Serotype 6B strain are all persistent opportunistic commensals of the human nasopharynx and are considered as the leading cause of community-acquired pneumonia. Understanding the molecular mechanisms of competition among these strains and the role of bacteriocins in these interactions is crucial in developing strategies to prevent and treat pneumococcal infections.

It has been previously proposed that the expression of bacteriocins, immunity, and the inducer peptide BlpC by *S. pneumoniae* are controlled by pH-dependent blp-promoters (Kjos et al., 2016). However, the present study has demonstrated that TIGR4 produced bacteriocin against *S. parasanguinis* under both slightly acidic (pH 6) and slightly alkaline (pH 8) conditions, suggesting that this activity is not pH-dependent as previously thought. However, a different producer strain P4 was found to only produce bacteriocin against P1104 at an alkaline pH 8 but not at pH 6. This is consistent with previous studies by Kjos et al. (2016) who found that most of the blp genes in *S. pneumoniae* D39 were not expressed under neutral conditions (pH 7.4) in the absence of the antibiotic kanamycin. This suggests that the competence regulatory system in pneumococcus is highly sensitive to environmental cues such as pH and certain antibiotics, and these cues may play a role in controlling the expression of bacteriocins and related genes.

We also investigated a putative immunity protein encoded in the TIGR4 *blp* locus. pBAV1K-T5 plasmids carrying a putative immunity gene within the blpMNO region was introduced into TIGR4. Expression was difficult to obtain, possibly because of toxicity of the expressed protein. However, the expression results on western blot and microscopy were conflicting. Therefore, further work is needed to investigate this anomaly and proceed to determine the functional effects of this protein.

Next, the present study evaluated the genetic basis of bacteriocin-resistance in *S. parasanguinis*, a Gram-positive bacterium, which is the primary commensal of the human oral cavity. Although this bacterium is known to be invasively pathogenic, it is associated with the development of dental plaque and infective endocarditis (Garnett et al., 2012). The bacteriocin resistance in *S. parasanguinis* followed a co-cultured with TIGR4 that produces Blp bacteriocin in BHI medium. High-quality DNA from bacteriocin-resistant and WT *S. parasanguinis* was sequenced, though no evidence of mutation was detected. Therefore, the resistance could be due to epigenetic changes, which expressed bacteriocin resistance trait. However, further studies are needed to re-evaluate any undetected inversions in the DNA sequence of the bacteriocin-resistant WT *S. parasanguinis*.

Finally, the present study evaluated the role of metal cations in pneumococcal bacteriocin inhibition. Several iron uptake systems have been discovered in *S. pneumoniae* and therefore, the present study investigated the role of metal-ion on bacteriocin production and whether metal-ion deficiency affects its ability to kill rival streptococcal species. The present study confirmed that Mn^{2+} and Fe^{3+} are essential for not only the growth of WT TIGR4, but also the bacteriocin-mediated inhibition of TIGR4 blp KO. Therefore, as key components of ABC transmembrane transporters, metal cations may play a role in stimulating the uptake/attachment of bacteriocins to the target cell. However, further experiments are warranted to differentiate

between a requirement of these ions for bacteriocin production or whether they are essential for bacteriocin action.

7.2 Implications of pneumococcal bacteriocins for clinical application

Pneumococcal bacteriocins have significant implications for medical applications, particularly as novel antimicrobial agents to replace conventional antibiotics in the near future. This development could have a significant impact on global public health in light of widespread antibiotic and multidrug resistance. Antimicrobial peptides (AMPs), such as bacteriocins, exhibit broad-spectrum activity against a wide range of bacterial, fungal, and viral pathogens including parasites. Importantly, AMPs are distinct from traditional antibiotics and possess unique mechanisms of action, which allows them to be used to treat a wide range of pathogens including multidrug-resistant strains. These mechanisms of action are less likely to be susceptible to resistance mechanisms commonly used by pathogenic bacteria to evade traditional antibiotics.

Recently, 14 new pneumococcal bacteriocin clusters have been discovered. Interestingly, identical or highly similar variants of the pneumococcal bacteriocin gene clusters have been characterized in other unrelated streptococcal species globally. This suggests that it may be possible to genetically transform less virulent commensals of the human nasopharynx, which are members of the streptococcal genus, into bacteriocin-producing strains by incorporating the blp locus. This can potentially be achieved by inoculating less virulent streptococcal species that lack the ability to cause invasive infection, with the blp locus.

7.3 Conclusion, Future Work and Perspective

The current understanding of the role of the blp locus in bacteriocin production in *S. pneumoniae* TIGR4 is limited and more research is needed to fully understand the molecular mechanisms behind it. Further studies should focus on identifying the specific genes and proteins that play the most important role in bacteriocin production and immunity in this

particular strain. The deletion of specific regions of the Blp locus can be accomplished using established methods that have been successfully used in targeting the entire locus. The analysis of bacteriocin production and activity can be done by extracting bacteria from high-density cultures and analysing the cell-free media for its ability to mediate bacterial inhibition, by 'spotting' onto lawns of susceptible bacteria. The mechanisms of bacterial inhibition can be further investigated by monitoring changes in cell wall permeability, which is believed to be one of the potential mechanisms by which pneumococcal bacteriocins exert their effects. One of the challenges in expressing recombinant bacteriocins is the hydrophobic nature of these molecules, which leads to auto-aggregation. To overcome this challenge, specific bacteriocins can be expressed as fusion proteins with a soluble protein such as thioredoxin using the pET102 vector. The bacteriocin can then be cleaved from the fusion protein using enterokinase and activity assayed in the solution. Additionally, studies should explore the possibility of heterodimer formation between different bacteriocin peptides as it is known that in Gram-positive bacteria, the activity of bacteriocins often requires heterodimer formation. This can be investigated by mixing different soluble bacteriocin peptides generated using the above methods, and then purifying the resulting heterodimers using standard protein chemistry techniques. This approach would likely result in more soluble heterodimers as modelling suggests that they interact using strong hydrophobic interactions, leaving hydrophilic residues exposed. Overall, a deeper understanding of the molecular mechanisms behind pneumococcal bacteriocins and their role in inter and intra-species competition could have important implications for medical applications and the development of novel antimicrobials as an alternative to conventional antibiotics. Thus, future similar studies should consider surveying the activity of pneumococcal bacteriocins against a range of different pathogenic bacteria.

References

Aguilar-Pérez, C., Gracia, B., Rodrigues, L., Vitoria, A., Cebrián, R., Deboosère, N., Song, O.R., Brodin, P., Maqueda, M. and Aínsa, J.A., (2018). Synergy between circular bacteriocin AS-48 and ethambutol against Mycobacterium tuberculosis. Antimicrobial agents and chemotherapy, 62(9), pp.e00359-18.

Al Aboud, N. M., Tupper, C., and Jialal, I. (2021). Genetics, Epigenetic Mechanism. In *Statpearls*. StatPearls Publishing Copyright © 2021, StatPearls Publishing LLC.

Al Atya, A.K., Belguesmia, Y., Chataigne, G., Ravallec, R., Vachée, A., Szunerits, S., Boukherroub, R. and Drider, D., (2016). Anti-MRSA activities of enterocins DD28 and DD93 and evidences on their role in the inhibition of biofilm formation. Frontiers in microbiology, 7, p.817.

AlKhatib, Z., Lagedroste, M., Fey, I., Kleinschrodt, D., Abts, A. and Smits, S.H., (2014). Lantibiotic immunity: inhibition of nisin mediated pore formation by NisI. *PloS one*, *9*(7), p.e102246.

Alves, L.A., de Carli, T.R., Harth-Chu, E.N., Mariano, F.S., Höfling, J.F., Stipp, R.N. and Mattos-Graner, R.O., (2019). Oral streptococci show diversity in resistance to complement immunity. *Journal of Medical Microbiology*, *68*(4), pp.600-608.

Amarasinghe, S. L., Su, S., Dong, X., Zappia, L., Ritchie, M. E., and Gouil, Q. (2020, 2020/02/07). Opportunities and Challenges in Long-Read Sequencing Data Analysis. *Genome Biology*, *21*(1), pp. 30.

Amin-Chowdhury, Z., Collins, S., Sheppard, C., Litt, D., Fry, N. K., Andrews, N., & Ladhani, S. N. (2020, Nov 5). Characteristics of invasive pneumococcal disease caused by emerging serotypes after the introduction of the 13-valent pneumococcal conjugate vaccine in england: A prospective observational cohort study, 2014-2018. *Clinical Infectious Diseases*, *71*(8), p.e235-e243.

Anderton, J.M., Rajam, G., Romero-Steiner, S., Summer, S., Kowalczyk, A.P., Carlone, G.M., Sampson, J.S. and Ades, E.W., (2007). E-cadherin is a receptor for the common protein pneumococcal surface adhesin A (PsaA) of Streptococcus pneumoniae. Microbial pathogenesis, 42(5-6), pp.225-236.

Andrews, S. (2010). *Fastqc: A Quality Control Tool for High Throughput Sequence Data*. http://www.bioinformatics.babraham.ac.uk/projects/fastqc

Arguedas, A., Trzciński, K., O'Brien, K.L., Ferreira, D.M., Wyllie, A.L., Weinberger, D., Danon, L., Pelton, S.I., Azzari, C., Hammitt, L.L. and Sá-Leão, R., (2020). Upper respiratory tract colonization with Streptococcus pneumoniae in adults. *Expert review of vaccines*, *19*(4), pp.353-366.

Balsells, E., Guillot, L., Nair, H. and Kyaw, M.H., 2017. Serotype distribution of Streptococcus pneumoniae causing invasive disease in children in the post-PCV era: a systematic review and meta-analysis. *PloS one*, *12*(5), p.e0177113.

Bauer, R., & Dicks, L. M. (2005, May 25). Mode of action of lipid II-targeting lantibiotics. *International Journal of Food Microbiology, 101*(2), p.201-216.

Bayle, L., Chimalapati, S., Schoehn, G., Brown, J., Vernet, T., & Durmort, C. (2011). Zinc Uptake by *Streptococcus pneumoniae* depends on both AdcA and AdcAII and is Essential for Normal Bacterial Morphology and Virulence. *Molecular microbiology*, *82*(4), 904-916.

Begde, D., Bundale, S., Mashitha, P., Rudra, J., Nashikkar, N. and Upadhyay, A., (2011). Immunomodulatory efficacy of nisin—a bacterial lantibiotic peptide. *Journal of peptide science*, *17*(6), pp.438-444.

Belguesmia, Y., Naghmouchi, K., Chihib, N.E. and Drider, D., (2011). Class IIa bacteriocins: current knowledge and perspectives. Prokaryotic antimicrobial peptides, pp.171-195.

Bhalla, M., Hui Yeoh, J., Lamneck, C., Herring, S. E., Tchalla, E. Y. I., Heinzinger, L. R., Leong, J. M., & Bou Ghanem, E. N. (2020, 2020/02/01). A1 adenosine receptor signaling reduces Streptococcus pneumoniae adherence to pulmonary epithelial cells by targeting expression of platelet-activating factor receptor [https://doi.org/10.1111/cmi.13141]. *Cellular Microbiology, 22*(2), p.e13141.

Blasi, F., Mantero, M., Santus, P. and Tarsia, P., (2012). Understanding the burden of pneumococcal disease in adults. *Clinical Microbiology and Infection*, *18*, pp.7-14.

Bogaardt, C., van Tonder, A. J., and Brueggemann, A. B. (2015). Genomic Analyses of Pneumococci Reveal a Wide Diversity of bacteriocins - Including Pneumocyclicin, a Novel Circular bacteriocin. *BMC genomics, 16*(1), pp. 554-554.

Bogaert, D., Keijser, B., Huse, S., Rossen, J., Veenhoven, R., van Gils, E., Bruin, J., Montijn, R., Bonten, M., and Sanders, E. (2011). Variability and Diversity of Nasopharyngeal Microbiota in Children: A Metagenomic Analysis. *PLOS ONE, 6*(2), pp. e17035.

Bolger, A. M., Lohse, M., and Usadel, B. (2014). Trimmomatic: A Flexible Trimmer for Illumina Sequence Data. *Bioinformatics, 30*(15), pp. 2114-2120.

Brand, A.M., Smith, C. and Dicks, L.M.T., 2013. Antimicrobial activity of nisin F, nisin A and Nisaplin® in the presence of serum and the effect of these lantibiotics on leukocyte functional capacity. *Therapeutic properties of the lantibiotic nisin F*. https://scholar.sun.ac.za/bitstream/handle/10019.1/79873/brand_therapeutic_2013.pdf?sequence=2&isAllowed=y#page=107

Brissac, T., Shenoy, A. T., Patterson, L. A., Orihuela, C. J., and Pirofski, L.-a. (2018). Cell Invasion and Pyruvate Oxidase-Derived H₂O₂ Are Critical for Streptococcus Pneumoniae-Mediated Cardiomyocyte Inhibition . *Infection and Immunity, 86*(1), pp. e00569-00517.

Brogden, K. A. (2005, 2005/03/01). Antimicrobial Peptides: Pore Formers or Metabolic Inhibitors in Bacteria? *Nature Reviews Microbiology, 3*(3), pp. 238-250.

Brown, E.J., Joiner, K.A., Cole, R.M. and Berger, M., (1983). Localization of complement component 3 on Streptococcus pneumoniae: anti-capsular antibody causes complement deposition on the pneumococcal capsule. Infection and Immunity, 39(1), pp.403-409.

Brown, J. S. (2012). Community-Acquired Pneumonia. *Clinical medicine (London, England), 12*(6), pp. 538-543.

Brown, L. R., Caulkins, R. C., Schartel, T. E., Rosch, J. W., Honsa, E. S., Schultz-Cherry, S., ... &

Bryksin, A. V., & Matsumura, I. (2010, Oct 8). Rational design of a plasmid origin that replicates efficiently in both gram-positive and gram-negative bacteria. *PLoS One, 5*(10), p.e13244.

Bryksin, A. V., and Matsumura, I. (2010). Rational Design of a Plasmid Origin That Replicates Efficiently in Both Gram-Positive and Gram-Negative Bacteria. *PLOS ONE, 5*(10), pp. e13244.

Bui, N.K., Eberhardt, A., Vollmer, D., Kern, T., Bougault, C., Tomasz, A., Simorre, J.P. and Vollmer, W., (2012). Isolation and analysis of cell wall components from Streptococcus pneumoniae. Analytical biochemistry, 421(2), pp.657-666.

Burcham, L.R. *et al.* (2020) *streptococcus pneumoniae metal homeostasis alters cellular metabolism, Metallomics: integrated biometal science*. U.S. National Library of Medicine.

Burghout, P., Cron, L. E., Gradstedt, H., Quintero, B., Simonetti, E., Bijlsma, J. J., ... & Hermans, P.W. (2010). Carbonic Anhydrase is Essential for *Streptococcus*

pneumoniae Growth in Environmental Ambient Air. *Journal of Bacteriology*, *192*(15), 4054-4062.

Bushell, B. (2021). *Bbmap Short Read Aligner, and Other Bioinformatic Tools.* . Retrieved 7/9/2021 from https://sourceforge.net/projects/bbmap/

Camilli, R., D'Ambrosio, F., Del Grosso, M., de Araujo, F.P., Caporali, M.G., Del Manso, M., Gherardi, G., D'Ancona, F., Pantosti, A. and Pneumococcal Surveillance Group, (2017). Impact of pneumococcal conjugate vaccine (PCV7 and PCV13) on pneumococcal invasive diseases in Italian children and insight into evolution of pneumococcal population structure. *Vaccine*, *35*(35), pp.4587-4593.

Campelo, A. B., López-González, M. J., Escobedo, S., Janzen, T., Neves, A. R., Rodríguez, A., and Martínez, B. (2020, 2020-August-13). Mutations Selected after Exposure to bacteriocin Lcn972 Activate a Bce-Like Bacitracin Resistance Module in Lactococcus Lactis [Original Research]. *Frontiers in microbiology*, *11*(1805).

Cao, K., Lai, F., Zhao, X. L., Wei, Q. X., Miao, X. Y., Ge, R., He, Q. Y., and Sun, X. (2018, Jul 30). The Mechanism of Iron-Compensation for Manganese Deficiency of Streptococcus Pneumoniae. *J Proteomics*, *184*, pp. 62-70.

Cardoso, M.R.A., Nascimento-Carvalho, C.M., Ferrero, F., Berezin, E.N., Ruvinsky, R., Camargos, P.A., Sant'Anna, C.C., Brandileone, M.C.C., Maria de Fátima, P., Feris-Iglesias, J. and Maggi, R.S., (2008). Penicillin-resistant pneumococcus and risk of treatment failure in pneumonia. Archives of disease in childhood, 93(3), pp.221-225.

Centers for Disease Control and Prevention -CDC. (2023, 20/1/2023). *Pneumococcal Vaccination: What Everyone Should Know*. cdc.gov. Retrieved 28/4/2023 from https://www.cdc.gov/vaccines/vpd/pneumo/public/index.html

Charpentier, X., Polard, P., and Claverys, J. P. (2012, Oct). Induction of Competence for Genetic Transformation by Antibiotics: Convergent Evolution of Stress Responses in Distant Bacterial Species Lacking Sos? *Curr Opin Microbiol, 15*(5), pp. 570-576.

Chaudhary, J. and Munshi, M., (1995). Scanning electron microscopic analysis of breast aspirates. Cytopathology, 6(3), pp.162-167.

Chen, K., Zhang, X., Shan, W., Zhao, G. and Zhang, T., (2018). Serotype distribution of Streptococcus pneumoniae and potential impact of pneumococcal conjugate vaccines in China: a systematic review and meta-analysis. *Human Vaccines & Immunotherapeutics*, *14*(6), pp.1453-1463.

Cheng, M. P., Bogoch, I. I., Green, K., Plevneshi, A., Rudnick, W., Shigayeva, A., McGeer, A., Lee, T. C., and Toronto Invasive Bacterial Diseases, N. (2018). Factors Associated with 30-Day Mortality Rate in Respiratory Infections Caused by Streptococcus Pneumoniae. *Clinical Infectious Diseases, 66*(8), pp. 1282-1285.

Chewapreecha, C., Harris, S. R., Croucher, N. J., Turner, C., Marttinen, P., Cheng, L., Pessia, A., Aanensen, D. M., Mather, A. E., Page, A. J., Salter, S. J., Harris, D., Nosten, F., Goldblatt, D., Corander, J., Parkhill, J., Turner, P., and Bentley, S. D. (2014, 2014/03/01). Dense Genomic Sampling Identifies Highways of Pneumococcal Recombination. *Nature Genetics, 46*(3), pp. 305-309.

Chien, Y.-W., Vidal, J. E., Grijalva, C. G., Bozio, C., Edwards, K. M., Williams, J. V., Griffin, M. R., Verastegui, H., Hartinger, S. M., Gil, A. I., Lanata, C. F., and Klugman, K. P. (2013). Density Interactions among Streptococcus Pneumoniae, Haemophilus Influenzae and Staphylococcus Aureus in the Nasopharynx of Young Peruvian Children. *The Pediatric infectious disease journal, 32*(1), pp. 72-77.

Chikindas, M.L., Weeks, R., Drider, D., Chistyakov, V.A. and Dicks, L.M., (2018). Functions and emerging applications of bacteriocins. Current opinion in biotechnology, 49, pp.23-28.

Christensen, D.P. and Hutkins, R.W., (1992). Collapse of the proton motive force in Listeria monocytogenes caused by a bacteriocin produced by Pediococcus acidilactici. *Applied and environmental microbiology*, *58*(10), pp.3312-3315.

Claverys, J. P., Martin, B., and Håvarstein, L. S. (2007, Jun). Competence-Induced Fratricide in Streptococci. *Mol Microbiol, 64*(6), pp. 1423-1433.

Cook, L. C., & Federle, M. J. (2014, May). Peptide pheromone signaling in Streptococcus and Enterococcus. *FEMS Microbiology Reviews 38*(3), pp.473-492.

Cotter, P. D., Hill, C., and Ross, R. P. (2005, Feb). Bacterial Lantibiotics: Strategies to Improve Therapeutic Potential. *Curr Protein Pept Sci, 6*(1), pp. 61-75.

Couvigny, B., de Wouters, T., Kaci, G., Jacouton, E., Delorme, C., Doré, J., Renault, P., Blottière, H. M., Guédon, E., and Lapaque, N. (2015). Commensal Streptococcus Salivarius Modulates Pparγ Transcriptional Activity in Human Intestinal Epithelial Cells. *PloS one*, *10*(5), pp. e0125371-e0125371.

Cremers, A.J., Zomer, A.L., Gritzfeld, J.F., Ferwerda, G., van Hijum, S.A., Ferreira, D.M., Shak, J.R., Klugman, K.P., Boekhorst, J., Timmerman, H.M. and de Jonge, M.I., (2014). The adult nasopharyngeal microbiome as a determinant of pneumococcal acquisition. *Microbiome*, *2*(1), pp.1-10.

Croucher, N. J., Harris, S. R., Fraser, C., Quail, M. A., Burton, J., van der Linden, M., McGee, L., von Gottberg, A., Song, J. H., Ko, K. S., Pichon, B., Baker, S., Parry, C. M., Lambertsen, L. M., Shahinas, D., Pillai, D. R., Mitchell, T. J., Dougan, G., Tomasz, A., Klugman, K. P., Parkhill, J., Hanage, W. P., and Bentley, S. D. (2011, Jan 28).

Rapid Pneumococcal Evolution in Response to Clinical Interventions. *Science, 331*(6016), pp. 430-434.

Dagkessamanskaia, A., Moscoso, M., Hénard, V., Guiral, S., Overweg, K., Reuter, M., Martin, B., Wells, J., and Claverys, J. P. (2004, Feb). Interconnection of Competence, Stress and Ciar Regulons in Streptococcus Pneumoniae: Competence Triggers Stationary Phase Autolysis of Ciar Mutant Cells. *Mol Microbiol, 51*(4), pp. 1071-1086.

Dalia, A. B., and Dalia, T. N. (2019). Horizontal Gene Transfer by Natural Transformation Promotes Both Genetic and Epigenetic Inheritance of Traits. *bioRxiv*, pp. 596379.

Daniels, C. C., Rogers, P. D., & Shelton, C. M. (2016, Jan-Feb). A Review of Pneumococcal Vaccines: Current Polysaccharide Vaccine Recommendations and Future Protein Antigens. *ournal of Pediatric Pharmacology and Therapeutics, 21*(1), pp.27-35.

Daningrat, W. O. D., Amalia, H., Ayu, I. M., Satzke, C., & Safari, D. (2022, 2022/02/01/). Carriage of Streptococcus pneumoniae in children under five years of age prior to pneumococcal vaccine introduction in Southeast Asia: A systematic review and meta-analysis (2001–2019). *Journal of Microbiology, Immunology and Infection, 55*(1), pp.6-17.

Danishyar, A. and Ashurst, J.V., (2017). Acute otitis media. https://europepmc.org/article/NBK/nbk470332#impact

Darbandi, A., Asadi, A., Mahdizade Ari, M., Ohadi, E., Talebi, M., Halaj Zadeh, M., Darb Emamie, A., Ghanavati, R., & Kakanj, M. (2022a, Jan). Bacteriocins: Properties and potential use as antimicrobials. *Journal of Clinical Laboratory Analysis 36*(1), p.e24093.

Darbandi, A., Asadi, A., Mahdizade Ari, M., Ohadi, E., Talebi, M., Halaj Zadeh, M., Darb Emamie, A., Ghanavati, R., & Kakanj, M. (2022b, Jan). Bacteriocins: Properties and

potential use as antimicrobials. *Journal of Clinical Laboratory Analysis, 36*(1), p.e24093.

Das, N., Valjavec-Gratian, M., Basuray, A. N., Fekete, R. A., Papp, P. P., Paulsson, J., and Chattoraj, D. K. (2005, Feb 22). Multiple Homeostatic Mechanisms in the Control of P1 Plasmid Replication. *Proc Natl Acad Sci U S A, 102*(8), pp. 2856-2861.

Dawid, S., Roche, A. M., & Weiser, J. N. (2007, Jan). The blp bacteriocins of Streptococcus pneumoniae mediate intraspecies competition both in vitro and in vivo. *Infection and Immunity, 75*(1), pp.443-451.

Dawid, S., Roche, A. M., and Weiser, J. N. (2007). The *Blp* bacteriocins of *Streptococcus Pneumoniae* Mediate Intraspecies Competition Both in Vitro and in Vivo. *Infection and Immunity, 75*(1), pp. 443-451.

de Cellès, M.D., Arduin, H., Lévy-Bruhl, D., Georges, S., Souty, C., Guillemot, D., Watier, L. and Opatowski, L., (2019). Unraveling the seasonal epidemiology of pneumococcus. Proceedings of the National Academy of Sciences, 116(5), pp.1802-1807.

de Saizieu, A., Gardès, C., Flint, N., Wagner, C., Kamber, M., Mitchell, T. J., Keck, W., Amrein, K. E., and Lange, R. (2000, Sep). Microarray-Based Identification of a Novel Streptococcus Pneumoniae Regulon Controlled by an Autoinduced Peptide. *J Bacteriol, 182*(17), pp. 4696-4703.

de Saizieu, A., Gardès, C., Flint, N., Wagner, C., Kamber, M., Mitchell Timothy, J., Keck, W., Amrein Kurt, E., & Lange, R. (2000, 2000/09/01). Microarray-Based Identification of a NovelStreptococcus pneumoniae Regulon Controlled by an Autoinduced Peptide. *Journal of Bacteriology*, *182*(17), pp.4696-4703.

De Stoppelaar, S.F., Bootsma, H.J., Zomer, A., Roelofs, J.J., Hermans, P.W., van't Veer, C. and van der Poll, T., (2013). Streptococcus pneumoniae serine protease HtrA, but not SFP or PrtA, is a major virulence factor in pneumonia. *PloS one, 8*(11), p.e80062.

defend against Oxidative Stress and Metal Ion Toxicity. *Journal of Bacteriology*, *194*(22), 6248-6254.

Denkovskienė, E., Paškevičius, Š., Misiūnas, A., Stočkūnaitė, B., Starkevič, U., Vitkauskienė, A., Hahn-Löbmann, S., Schulz, S., Giritch, A., Gleba, Y. and Ražanskienė, A., (2019). Broad and efficient control of Klebsiella pathogens by peptidoglycan-degrading and pore-forming bacteriocins klebicins. Scientific reports, 9(1), pp.1-11.

Dicks, L.M., Dreyer, L., Smith, C. and Van Staden, A.D., (2018). A review: the fate of bacteriocins in the human gastro-intestinal tract: do they cross the gut–blood barrier?. *Frontiers in Microbiology*, p.2297.

Dieudonné-Vatran, A., Krentz, S., Blom, A.M., Meri, S., Henriques-Normark, B., Riesbeck, K. and Albiger, B., (2009). Clinical isolates of Streptococcus pneumoniae bind the complement inhibitor C4b-binding protein in a PspC allele-dependent fashion. The Journal of Immunology, 182(12), pp.7865-7877.

Dinh, A., Duran, C., Ropers, J., Bouchand, F., Davido, B., Deconinck, L., Matt, M., Senard, O., Lagrange, A., Mellon, G. and Calin, R., (2021). Factors associated with treatment failure in moderately severe community-acquired pneumonia: a secondary analysis of a randomized clinical trial. *JAMA network open*, *4*(10), pp.e2129566-e2129566.

Dobay, O. (2019, 2019/11/02). The Complexity of Serotype Replacement of Pneumococci. *Human Vaccines & Immunotherapeutics*, *15*(11), pp. 2725-2728.

Donkor, E. S., Bishop, C. J., Gould, K., Hinds, J., Antonio, M., Wren, B., and Hanage, W. P. (2011). High Levels of Recombination among Streptococcus Pneumoniae Isolates from the Gambia. *mBio, 2*(3), pp. e00040-00011.

Drider, D., Bendali, F., Naghmouchi, K. and Chikindas, M.L., (2016). bacteriocins: not only antibacterial agents. Probiotics and antimicrobial proteins, 8(4), pp.177-182.

Drissi, F., Buffet, S., Raoult, D., & Merhej, V. (2015). Common occurrence of antibacterial agents in human intestinal microbiota. *Frontiers in Microbiology, 6*(May 7), p.441.

Duan, F., Li, X., Cai, S., Xin, G., Wang, Y., Du, D., He, S., Huang, B., Guo, X., Zhao, H. and Zhang, R., (2014). Haloemodin as novel antibacterial agent inhibiting DNA gyrase and bacterial topoisomerase I. *Journal of medicinal chemistry*, *57*(9), pp.3707-3714.

Durmort, C., Ercoli, G., Ramos-Sevillano, E., Chimalapati, S., Haigh, R. D., Croix, M. D. S., Gould, K., Hinds, J., Guerardel, Y., Vernet, T., Oggioni, M., Brown, J. S., Keller, L. E., and McDaniel, L. S. (2020). Deletion of the Zinc Transporter Lipoprotein Adcaii Causes Hyperencapsulation of Streptococcus Pneumoniae Associated with Distinct Alleles of the Type I Restriction-Modification System. *mBio, 11*(2), pp. e00445-00420.

Durrett, R. and Levin, S., (1997). Allelopathy in spatially distributed populations. *Journal of theoretical biology*, *185*(2), pp.165-171.

Eijkelkamp, B. A., Morey, J. R., Ween, M. P., Ong, C.-l. Y., McEwan, A. G., Paton, J. C., and McDevitt, C. A. (2014). Extracellular Zinc Competitively Inhibits Manganese Uptake and Compromises Oxidative Stress Management in Streptococcus Pneumoniae. *PloS one, 9*(2), pp. e89427-e89427.

Eijsink, V. G., Axelsson, L., Diep, D. B., Håvarstein, L. S., Holo, H., and Nes, I. F. (2002, Aug). Production of Class Ii bacteriocins by Lactic Acid Bacteria; an Example of Biological Warfare and Communication. *Antonie Van Leeuwenhoek, 81*(1-4), pp. 639-654.

El-Gewely, M. R., Fenton, C., Kjeldsen, E., and Xu, H. Mutagenesis: Site-Specific. In *Els*.

Engen, S. A., Valen Rukke, H., Becattini, S., Jarrossay, D., Blix, I. J., Petersen, F. C., Sallusto, F., & Schenck, K. (2014). The Oral Commensal Streptococcus mitis Shows a Mixed

Memory Th Cell Signature That Is Similar to and Cross-Reactive with Streptococcus pneumoniae. *PLoS One, 9*(8), p.e104306.

Engholm, D. H., Kilian, M., Goodsell, D. S., Andersen, E. S., and Kjærgaard, R. S. (2017). A Visual Review of the Human Pathogen Streptococcus Pneumoniae. *FEMS Microbiology Reviews, 41*(6), pp. 854-879.

Etayash, H., Azmi, S., Dangeti, R. and Kaur, K., (2016). Peptide bacteriocins-structure activity relationships. *Current topics in medicinal chemistry*, *16*(2), pp.220-241.

Ewing, B., and Green, P. (1998, Mar). Base-Calling of Automated Sequencer Traces Using Phred. Ii. Error Probabilities. *Genome Res, 8*(3), pp. 186-194.

Eythorsson, E., Hrafnkelsson, B., Erlendsdóttir, H., Gudmundsson, S.A., Kristinsson, K.G. and Haraldsson, Á., (2018). Decreased acute otitis media with treatment failure after introduction of the ten-valent pneumococcal Haemophilus influenzae protein D conjugate vaccine. *The Pediatric infectious disease journal*, *37*(4), pp.361-366.

Feikin, D.R., Kagucia, E.W., Loo, J.D., Link-Gelles, R., Puhan, M.A., Cherian, T., Levine, O.S., Whitney, C.G., O'brien, K.L., Moore, M.R. and Serotype Replacement Study Group, (2013). Serotype-specific changes in invasive pneumococcal disease after pneumococcal conjugate vaccine introduction: a pooled analysis of multiple surveillance sites. *PLoS medicine*, *10*(9), p.e1001517.

Feldman, C., Munro, N.C., Jeffery, P.K., Mitchell, T.J., Andrew, P.W., Boulnois, G.J., Guerreiro, D., Rohde, J.A., Todd, H.C., Cole, P.J. and Wilson, R., (1991). Pneumolysin induces the salient histologic features of pneumococcal infection in the rat lung in vivo. Am J Respir Cell Mol Biol, 5(5), pp.416-423.

Flasche, S., Van Hoek, A.J., Sheasby, E., Waight, P., Andrews, N., Sheppard, C., George, R. and Miller, E., (2011). Effect of pneumococcal conjugate vaccination on serotype-

specific carriage and invasive disease in England: a cross-sectional study. *PLoS medicine*, *8*(4), p.e1001017.

Fucile, G. et al. ePlant and the 3D data display initiative: integrative systems biology on the world wide web. *PLoS ONE* **6**, e15237 (2010).

Furgerson Ihnken, L. A., Chatterjee, C., & van der Donk, W. A. (2008, 2008/07/01). In Vitro Reconstitution and Substrate Specificity of a Lantibiotic Protease. *Biochemistry, 47*(28), pp7352-7363.

Gagne, A. L., Stevens, K. E., Cassone, M., Pujari, A., Abiola, O. E., Chang, D. J., & Sebert, M. E. (2013). Competence in Streptococcus pneumoniae is a response to an increasing mutational burden. *PLoS One, 8*(8), p.e72613.

Ganaie, F., Saad, J. S., McGee, L., Tonder, A. J. v., Bentley, S. D., Lo, S. W., Gladstone, R. A., Turner, P., Keenan, J. D., Breiman, R. F., & Nahm, M. H. (2020). A New Pneumococcal Capsule Type, 10D, is the 100th Serotype and Has a Large cps Fragment from an Oral Streptococcus. *mBio, 11*(3), p.e00937-00920.

Garcia-Bustos, J.F., Chait, B.T. and Tomasz, A., (1987). Structure of the peptide network of pneumococcal peptidoglycan. Journal of Biological Chemistry, 262(32), pp.15400-15405.

Garcia-Gutierrez, E., Mayer, M.J., Cotter, P.D. and Narbad, A., (2019). Gut microbiota as a source of novel antimicrobials. Gut microbes, 10(1), pp.1-21.

Garnett, J. A., Simpson, P. J., Taylor, J., Benjamin, S. V., Tagliaferri, C., Cota, E., Chen, Y.-Y. M., Wu, H., and Matthews, S. (2012). Structural Insight into the Role of Streptococcus Parasanguinis Fap1 within Oral Biofilm Formation. *Biochemical and biophysical research communications, 417*(1), pp. 421-426.

Geng, J., Chiu, C.-H., Tang, P., Chen, Y., Shieh, H.-R., Hu, S., and Chen, Y.-Y. M. (2012). Complete Genome and Transcriptomes of Streptococcus Parasanguinis Fw213:

Phylogenic Relations and Potential Virulence Mechanisms. *PLOS ONE, 7*(4), pp. e34769.

Geno, K.A., Saad, J.S. and Nahm, M.H., (2017). Discovery of novel pneumococcal serotype 35D, a natural WciG-deficient variant of serotype 35B. Journal of clinical microbiology, 55(5), pp.1416-1425.

Godman, B., Shrank, W., Wettermark, B., Andersen, M., Bishop, I., Burkhardt, T., Garuolienè, K., Kalaba, M., Laius, O., Joppi, R. and Sermet, C., (2010). Use of generics—a critical cost containment measure for all healthcare professionals in Europe?. Pharmaceuticals, 3(8), pp.2470-2494.

Gómez-Mejia, A., Gámez, G. and Hammerschmidt, S., (2018). Streptococcus pneumoniae two-component regulatory systems: The interplay of the pneumococcus with its environment. *International Journal of Medical Microbiology, 308*(6), pp.722-737.

Gratia, A. and Fredericq, P., (1946). Diversité des souches antibiotiques de B. coli et étendue variable de leur champ d'action. Comptes Rendus des Séances de la Société de Biologie et de ses Filiales, 140, pp. 1032–1033.

Griffith, F. (1928, Jan). The Significance of Pneumococcal Types. *J Hyg (Lond), 27*(2), pp. 113-159.

Guiral, S., Mitchell, T. J., Martin, B., and Claverys, J.-P. (2005). Competence- programd Predation of Noncompetent Cells in the Human Pathogen ≪Em≫Streptococcus Pneumoniae≪/Em≫: Genetic Requirements. *Proceedings of the National Academy of Sciences of the United States of America, 102*(24), pp. 8710.

Güllüce, M., Karadayı, M., and Bariş, Ö. (2013). bacteriocins: Promising Natural Antimicrobials. In.

Gurevich, A., Saveliev, V., Vyahhi, N., and Tesler, G. (2013). Quast: Quality Assessment Tool for Genome Assemblies. *Bioinformatics, 29*(8), pp. 1072-1075.

Gutiérrez-Fernández, J., Saleh, M., Alcorlo, M., Gómez-Mejía, A., Pantoja-Uceda, D., Treviño, M.A., Voß, F., Abdullah, M.R., Galán-Bartual, S., Seinen, J. and Sánchez-Murcia, P.A., (2016). Modular architecture and unique teichoic acid recognition features of choline-binding protein L (CbpL) contributing to pneumococcal pathogenesis. *Scientific reports*, *6*(1), pp.1-19.

Hamaguchi, S., Zafar, M.A., Cammer, M. and Weiser, J.N., (2018). Capsule prolongs survival of Streptococcus pneumoniae during starvation. *Infection and immunity*, *86*(3), pp.e00802-17.

Hanage, W.P., Finkelstein, J.A., Huang, S.S., Pelton, S.I., Stevenson, A.E., Kleinman, K., Hinrichsen, V.L. and Fraser, C., (2010). Evidence that pneumococcal serotype replacement in Massachusetts following conjugate vaccination is now complete. *Epidemics*, *2*(2), pp.80-84.

Harth-Chu, E. N., Alves, L. A., Theobaldo, J. D., Salomão, M. F., Höfling, J. F., King, W. F., Smith, D. J., & Mattos-Graner, R. O. (2019). PcsB Expression Diversity Influences on Streptococcus mitis Phenotypes Associated With Host Persistence and Virulence. *Frontiers in Microbiology*, *10*(Nov 12), p.2567.

Hathaway, L. J., Brugger, S. D., Morand, B., Bangert, M., Rotzetter, J. U., Hauser, C., Graber, W. A., Gore, S., Kadioglu, A., and Mühlemann, K. (2012). Capsule Type of Streptococcus Pneumoniae Determines Growth Phenotype. *PLOS Pathogens*, *8*(3), pp. e1002574.

Håvarstein, L. S., Diep, D. B., and Nes, I. F. (1995, Apr). A Family of bacteriocin Abc Transporters Carry out Proteolytic Processing of Their Substrates Concomitant with Export. *Mol Microbiol*, *16*(2), pp. 229-240.

Håvarstein, L. S., Martin, B., Johnsborg, O., Granadel, C., and Claverys, J. P. (2006, Feb). New Insights into the Pneumococcal Fratricide: Relationship to Clumping and Identification of a Novel Immunity Factor. *Mol Microbiol, 59*(4), pp. 1297-1307.

Héchard, Y. and Sahl, H.G., (2002). Mode of action of modified and unmodified bacteriocins from Gram-positive bacteria. *Biochimie*, *84*(5-6), pp.545-557.

Heng, L. (2020). *Aun: A New Metric to Measure Assembly Contiguity* Retrieved 7/9/2021 from https://lh3.github.io/2020/04/08/a-new-metric-on-assembly-contiguity

Hermans, P.W., Adrian, P.V., Albert, C., Estevão, S., Hoogenboezem, T., Luijendijk, I.H., Kamphausen, T. and Hammerschmidt, S., (2006). The streptococcal lipoprotein rotamase A (SlrA) is a functional peptidyl-prolyl isomerase involved in pneumococcal colonization. *Journal of Biological Chemistry*, *281*(2), pp.968-976.

Hernández-González, J. C., Martínez-Tapia, A., Lazcano-Hernández, G., García-Pérez, B. E., and Castrejón-Jiménez, N. S. (2021, Apr 1). bacteriocins from Lactic Acid Bacteria. A Powerful Alternative as Antimicrobials, Probiotics, and Immunomodulators in Veterinary Medicine. *Animals (Basel), 11*(4).

Hernández-González, J. C., Martínez-Tapia, A., Lazcano-Hernández, G., García-Pérez, B. E., & Castrejón-Jiménez, N. S. (2021, Apr 1). Bacteriocins from Lactic Acid Bacteria. A Powerful Alternative as Antimicrobials, Probiotics, and Immunomodulators in Veterinary Medicine. *Animals (Basel), 11*(4).

Hiller, N. L., Ahmed, A., Powell, E., Martin, D. P., Eutsey, R., Earl, J., Janto, B., Boissy, R. J., Hogg, J., Barbadora, K., Sampath, R., Lonergan, S., Post, J. C., Hu, F. Z., and Ehrlich, G. D. (2010, Sep 16). Generation of Genic Diversity among Streptococcus Pneumoniae Strains Via Horizontal Gene Transfer During a Chronic Polyclonal Pediatric Infection. *PLoS Pathog, 6*(9), pp. e1001108.

Hjálmarsdóttir, M.Á., Gumundsdóttir, P.F., Erlendsdóttir, H., Kristinsson, K.G. and Haraldsson, G., (2016). Cocolonization of pneumococcal serotypes in healthy children attending day care centers: molecular versus conventional methods. *The Pediatric infectious disease journal, 35*(5), pp.477-480.

Högberg, L., Geli, P., Ringberg, H., Melander, E., Lipsitch, M., and Ekdahl, K. (2007, 04/01). Age- and Serogroup-Related Differences in Observed Durations of Nasopharyngeal Carriage of Penicillin-Resistant Pneumococci. *Journal of clinical microbiology, 45*, pp. 948-952.

Hols, P., Ledesma-García, L., Gabant, P. and Mignolet, J., (2019). Mobilization of microbiota commensals and their bacteriocins for therapeutics. *Trends in microbiology*, *27*(8), pp.690-702.

Hotchkiss, R. D. (1951). Transfer of Penicillin Resistance in Pneumococci by the Desoxyribonucleate Derived from Resistant Cultures. *Cold Spring Harb Symp Quant Biol, 16*, pp. 457-461.

Huang, F., Teng, K., Liu, Y., Cao, Y., Wang, T., Ma, C., Zhang, J., and Zhong, J. (2021, 2021/04/12). bacteriocins: Potential for Human Health. *Oxidative Medicine and Cellular Longevity, 2021*, pp. 5518825.

Ibrahim, O.O., (2019). Classification of antimicrobial peptides bacteriocins, and the nature of some bacteriocins with potential applications in food safety and bio-pharmaceuticals. EC Microbiol, 15(7), pp.591-608.

Ibrahim, Y.M., Kerr, A.R., McCluskey, J. and Mitchell, T.J., (2004). Role of HtrA in the virulence and competence of Streptococcus pneumoniae. *Infection and immunity*, *72*(6), pp.3584-3591.

Idalia, V.-M., and Franco, B. (2017). Escherichia Coli as a Model Organism and Its Application in Biotechnology. In.

Illumina Inc. (2021). *Understanding the Genetic Code*. https://emea.illumina.com/techniques/sequencing/dna-sequencing.html

Islam, M. R., Nagao, J., Zendo, T., and Sonomoto, K. (2012, Dec 1). Antimicrobial Mechanism of Lantibiotics. *Biochem Soc Trans, 40*(6), pp. 1528-1533.

Isturiz, R., Sings, H.L., Hilton, B., Arguedas, A., Reinert, R.R. and Jodar, L., (2017). Streptococcus pneumoniae serotype 19A: worldwide epidemiology. Expert review of vaccines, 16(10), pp.1007-1027.

Isturiz, R.E., Ramirez, J., Self, W.H., Grijalva, C.G., Counselman, F.L., Volturo, G., Ostrosky-Zeichner, L., Peyrani, P., Wunderink, R.G., Sherwin, R. and Overcash, J.S., (2019). Pneumococcal epidemiology among us adults hospitalized for community-acquired pneumonia. Vaccine, 37(25), pp.3352-3361.

Jacobs, M.R., Koornhof, H.J., Robins-Browne, R.M., Stevenson, C.M., Vermaak, Z.A., Freiman, I., Miller, G.B., Witcomb, M.A., Isaäcson, M., Ward, J.I. and Austrian, R., (1978). Emergence of multiply resistant pneumococci. *New England Journal of Medicine*, *299*(14), pp.735-740.

Jain, A., and Srivastava, P. (2013). Broad Host Range Plasmids. *FEMS Microbiology Letters, 348*(2), pp. 87-96.

Janoir, C., Lepoutre, A., Gutmann, L. and Varon, E., (2016). Insight into resistance phenotypes of emergent non-PCV13 type pneumococci isolated from invasive disease after PCV13 implementation in France. In *Open Forum Infect Dis*, *3*(1), 1–9.

Jiang, H., Zou, J., Cheng, H., Fang, J. and Huang, G., (2017). Purification, characterization, and mode of action of pentocin JL-1, a novel bacteriocin isolated from Lactobacillus pentosus, against drug-resistant Staphylococcus aureus. BioMed Research International, 2017.

Jin, B., Li, Y., and Robertson, K. D. (2011). DNA Methylation: Superior or Subordinate in the Epigenetic Hierarchy? *Genes & cancer*, *2*(6), pp. 607-617.

John, C.C., (1994). Treatment failure with use of a third-generation cephalosporin for penicillin-resistant pneumococcal meningitis: case report and review. *Clinical infectious diseases*, *18*(2), pp.188-193.

Jolley, K. A., Bliss, C. M., Bennett, J. S., Bratcher, H. B., Brehony, C., Colles, F. M., Wimalarathna, H., Harrison, O. B., Sheppard, S. K., Cody, A. J., and Maiden, M. C. J. (2012, Apr). Ribosomal Multilocus Sequence Typing: Universal Characterization of Bacteria from Domain to Strain. *Microbiology (Reading)*, *158*(Pt 4), pp. 1005-1015.

Kadioglu, A., Weiser, J. N., Paton, J. C., and Andrew, P. W. (2008, Apr). The Role of Streptococcus Pneumoniae Virulence Factors in Host Respiratory Colonization and Disease. *Nat Rev Microbiol*, *6*(4), pp. 288-301.

Kamng'ona, A.W., Hinds, J., Bar-Zeev, N., Gould, K.A., Chaguza, C., Msefula, C., Cornick, J.E., Kulohoma, B.W., Gray, K., Bentley, S.D. and French, N., (2015). High multiple carriage and emergence of Streptococcus pneumoniae vaccine serotype variants in Malawian children. *BMC infectious diseases*, *15*(1), pp.1-11.

Kartasasmita, C.B., Rezeki Hadinegoro, S., Kurniati, N., Triasih, R., Halim, C. and Gamil, A., (2020). Epidemiology, nasopharyngeal carriage, serotype prevalence, and antibiotic resistance of streptococcus pneumoniae in Indonesia. *Infectious Diseases and Therapy*, *9*(4), pp.723-736.

Kaur, I. P., & Deol, P. K. (2020). *Probiotic Research in Therapeutics: Volume 1: Applications in Cancers and Immunological Diseases*. Springer Nature Singapore.

Kaur, S., & Kaur, S. (2015). Bacteriocins as potential anticancer agents. *Frontiers in Pharmacology*, *6*(Nov 10), p.272.

Kavalari, I.D., Fuursted, K., Krogfelt, K.A. and Slotved, H.C., (2019). Molecular characterization and epidemiology of Streptococcus pneumoniae serotype 24F in Denmark. *Scientific reports*, *9*(1), pp.1-9.

Kawe, M., Horn, U., and Plückthun, A. (2009). Facile Promoter Deletion in Escherichia Coli in Response to Leaky Expression of Very Robust and Benign Proteins from Common Expression Vectors. *Microbial cell factories, 8*, pp. 8-8.

Kerr, B., Riley, M.A., Feldman, M.W. and Bohannan, B.J., (2002). Local dispersal promotes biodiversity in a real-life game of rock–paper–scissors. *Nature, 418*(6894), pp.171-174.

Kilian, M. and Tettelin, H., (2019). Identification of virulence-associated properties by comparative genome analysis of Streptococcus pneumoniae, S. pseudopneumoniae, S. mitis, three S. oralis subspecies, and S. infantis. *MBio, 10*(5), pp.e01985-19.

Kilian, M., Poulsen, K., Blomqvist, T., Håvarstein, L.S., Bek-Thomsen, M., Tettelin, H. and Sørensen, U.B., (2008). Evolution of Streptococcus pneumoniae and its close commensal relatives. *PloS one*, *3*(7), p.e2683.

Kilian, M., Riley, D.R., Jensen, A., Brüggemann, H. and Tettelin, H., (2014). Parallel evolution of Streptococcus pneumoniae and Streptococcus mitis to pathogenic and mutualistic lifestyles. *MBio, 5*(4), pp.e01490-14.

Kim, E.H., Choi, S.Y., Kwon, M.K., Tran, T.D.H., Park, S.S., Lee, K.J., Bae, S.M., Briles, D.E. and Rhee, D.K., (2012). Streptococcus pneumoniae pep27 mutant as a live vaccine for serotype-independent protection in mice. Vaccine, 30(11), pp.2008-2019.

Kjos, M., Miller, E., Slager, J., Lake, F. B., Gericke, O., Roberts, I. S., Rozen, D. E., and Veening, J.-W. (2016). Expression of Streptococcus Pneumoniae bacteriocins Is Induced by Antibiotics Via Regulatory Interplay with the Competence System. *PLOS Pathogens, 12*(2), pp. e1005422.

Kjos, M., Miller, E., Slager, J., Lake, F.B., Gericke, O., Roberts, I.S., Rozen, D.E. and Veening, J.W., (2016). Expression of Streptococcus pneumoniae bacteriocins is induced by antibiotics via regulatory interplay with the competence system. PLoS pathogens, 12(2), p.e1005422.

Kochan, T. J., and Dawid, S. (2013). The Htra Protease of Streptococcus Pneumoniae Controls Density-Dependent Stimulation of the bacteriocin Blp Locus Via Disruption of Pheromone Secretion. *Journal of Bacteriology, 195*(7), pp. 1561-1572.

Koelman, D.L.H., Brouwer, M.C. and van de Beek, D., (2020). Resurgence of pneumococcal meningitis in Europe and Northern America. Clinical microbiology and infection, 26(2), pp.199-204.

Kommineni, S., Bretl, D.J., Lam, V., Chakraborty, R., Hayward, M., Simpson, P., Cao, Y., Bousounis, P., Kristich, C.J. and Salzman, N.H., (2015). bacteriocin production augments niche competition by enterococci in the mammalian gastrointestinal tract. Nature, 526(7575), pp.719-722.

Kono, M., Zafar, M.A., Zuniga, M., Roche, A.M., Hamaguchi, S. and Weiser, J.N., (2016). Single cell bottlenecks in the pathogenesis of Streptococcus pneumoniae. *PLoS pathogens, 12*(10), p.e1005887.

Könönen, E., Jousimies-Somer, H., Bryk, A., Kilpi, T., and Kilian, M. (2002, Sep). Establishment of Streptococci in the Upper Respiratory Tract: Longitudinal Changes in the Mouth and Nasopharynx up to 2 Years of Age. *J Med Microbiol, 51*(9), pp. 723-730.

Kościuczuk, E. M., Lisowski, P., Jarczak, J., Strzałkowska, N., Jóźwik, A., Horbańczuk, J., Krzyżewski, J., Zwierzchowski, L., and Bagnicka, E. (2012, Dec). Cathelicidins: Family of Antimicrobial Peptides. A Review. *Mol Biol Rep, 39*(12), pp. 10957-10970.

Kotake, Y., Ishii, S., Yano, T., Katsuoka, Y., and Hayashi, H. (2008, Feb 26). Substrate Recognition Mechanism of the Peptidase Domain of the Quorum-Sensing-Signal-Producing Abc Transporter Coma from Streptococcus. *Biochemistry, 47*(8), pp. 2531-2538.

Kristinsson, K.G., (1999). Modification of prescribers' behavior: the Icelandic approach. *Clinical Microbiology and infection*, *5*, pp.4S43-4S47.

Krzyściak, W., Pluskwa, K. K., Jurczak, A., and Kościelniak, D. (2013). The Pathogenicity of the Streptococcus Genus. *European journal of clinical microbiology & infectious diseases : official publication of the European Society of Clinical Microbiology, 32*(11), pp. 1361-1376.

Kumariya, R., Garsa, A.K., Rajput, Y.S., Sood, S.K., Akhtar, N. and Patel, S., (2019). Classification, synthesis, mechanism of action and resistance development in food spoilage causing bacteria. *Microbial pathogenesis*, *128*, pp.171-177.

Kunkel, T. A. (1985, Jan). Rapid and Efficient Site-Specific Mutagenesis without Phenotypic Selection. *Proc Natl Acad Sci U S A, 82*(2), pp. 488-492.

Kuo, C.-J., Lee, C.-H., Chang, M.-L., Lin, C.-Y., Lin, W.-R., Su, M.-Y., Chiu, C.-H., Tseng, C.-N., Wu, Y.-S., Chiu, C.-T., and Lai, C.-H. (2021, 2021/03/23/). Multidrug Resistance: The Clinical Dilemma of Refractory Helicobacter Pylori Infection. *Journal of Microbiology, Immunology and Infection*.

Ladhani, S.N., Collins, S., Djennad, A., Sheppard, C.L., Borrow, R., Fry, N.K., Andrews, N.J., Miller, E. and Ramsay, M.E., (2018). Rapid increase in non-vaccine serotypes causing invasive pneumococcal disease in England and Wales, 2000–17: a prospective national observational cohort study. *The Lancet infectious diseases*, *18*(4), pp.441-451.

Lee, K., Walker, A.R., Chakraborty, B., Kaspar, J.R., Nascimento, M.M. and Burne, R.A., (2019). Novel probiotic mechanisms of the oral bacterium Streptococcus sp. A12 as explored with functional genomics. Applied and environmental microbiology, 85(21), pp.e01335-19.

Lehtinen, S., Croucher, N.J., Blanquart, F. and Fraser, C., (2020). Epidemiological dynamics of bacteriocin competition and antibiotic resistance. *bioRxiv*.

Levin, B.R., (1988). Frequency-dependent selection in bacterial populations. *Philosophical Transactions of the Royal Society of London. B, Biological Sciences*, *319*(1196), pp.459-472.

Lewis, T.E. et al. Genome3D: a UK collaborative project to annotate genomic sequences with predicted 3D structures based on SCOP and CATH domains. *Nucl. Acids Res.* **41**, D499–D507 (2013).

Lewnard, J.A. and Hanage, W.P., (2019). Making sense of differences in pneumococcal serotype replacement. *The Lancet Infectious Diseases*, *19*(6), pp.e213-e220.

Lewnard, J.A., Tähtinen, P.A., Laine, M.K., Lindholm, L., Jalava, J., Huovinen, P., Lipsitch, M. and Ruohola, A., (2018). Impact of antimicrobial treatment for acute otitis media on carriage dynamics of penicillin-susceptible and penicillin-nonsusceptible Streptococcus pneumoniae. *The Journal of infectious diseases*, *218*(9), pp.1356-1366.

Li, Y., Weinberger, D. M., Thompson, C. M., Trzciński, K., and Lipsitch, M. (2013, Dec). Surface Charge of Streptococcus Pneumoniae Predicts Serotype Distribution. *Infect Immun, 81*(12), pp. 4519-4524.

Lipsitch, M. and Moxon, E.R., 1997. Virulence and transmissibility of pathogens: what is the relationship?. *Trends in microbiology*, *5*(1), pp.31-37.

Lobo-Ruiz, A. and Tulla-Puche, J., (2018). Synthetic approaches of naturally and rationally designed peptides and peptidomimetics. In Peptide applications in biomedicine, biotechnology and bioengineering (pp. 23-49). Woodhead Publishing.

Lovino, F., Molema, G. and Bijlsma, J.J., (2014). Streptococcus pneumoniae interacts with pIgR expressed by the brain microvascular endothelium but does not co-localize with PAF receptor. *PLoS One*, *9*(5), p.e97914.

Lovino, F., Seinen, J., Henriques-Normark, B. and van Dijl, J.M., (2016). How does Streptococcus pneumoniae invade the brain?. *Trends in microbiology*, *24*(4), pp.307-315.

Lux, T., Nuhn, M., Hakenbeck, R., and Reichmann, P. (2007, 2007/11/01). Diversity of bacteriocins and Activity Spectrum in Streptococcus Pneumoniae. *Journal of Bacteriology*, *189*(21), pp. 7741-7751.

Mahlapuu, M., Håkansson, J., Ringstad, L., and Björn, C. (2016). Antimicrobial Peptides: An Emerging Category of Therapeutic Agents. *Frontiers in cellular and infection microbiology*, *6*, pp. 194-194.

Maldonado-Barragan A., Cardenas, N., Martinez, B., Ruiz-Barba, J. L., Fernandez-Garayzabal, J. F., Rodrïguez, J. M. and Gibello, A., 2013. Garvicin A, a novel class IId bacteriocin from Lactococcus garvieae that inhibits septum formation in L. garvieae strains. *Applied and environmental microbiology*, *79*(14), pp.4336-4346.

Manna, S., Harman, A., Accari, J., and Barth, C. (2013, Mar 6). Altering the Selection Capabilities of Common Cloning Vectors Via Restriction Enzyme Mediated Gene Disruption. *BMC Res Notes*, *6*, pp. 85.

Manning, J., Dunne, E.M., Wescombe, P.A., Hale, J.D., Mulholland, E.K., Tagg, J.R., Robins-Browne, R.M. and Satzke, C., (2016). Investigation of Streptococcus salivarius-

mediated inhibition of pneumococcal adherence to pharyngeal epithelial cells. BMC microbiology, 16(1), pp.1-9.

Mao, C. et al. Functional assignment of *Mycobacterium tuberculosis* proteome by genome-scale fold-recognition. *Tuberculosis* **1**, 93 (2013).

Marchisio, P., Galli, L., Bortone, B., Ciarcià, M., Motisi, M.A., Novelli, A., Pinto, L., Bottero, S., Pignataro, L., Piacentini, G. and Mattina, R., (2019). Updated guidelines for the management of acute otitis media in children by the Italian Society of Pediatrics: treatment. *The Pediatric infectious disease journal*, *38*(12S), pp.S10-S21.

Maricic, N., Anderson, E.S., Opipari, A.E., Yu, E.A. and Dawid, S., (2016). Characterization of a multipeptide lantibiotic locus in Streptococcus pneumoniae. MBio, 7(1), pp.e01656-15.

Marquart, M.E., (2021). Pathogenicity and virulence of Streptococcus pneumoniae: Cutting to the chase on proteases. Virulence, 12(1), pp.766-787.

Masomian, M., Ahmad, Z., Ti Gew, L. and Poh, C.L., (2020). Development of next generation Streptococcus pneumoniae vaccines conferring broad protection. Vaccines, 8(1), p.132.

Matevosyan, L., Bazukyan, I., and Trchounian, A. (2019). Comparative Analysis of the Effect of Ca and Mg Ions on Antibacterial Activity of Lactic Acid Bacteria Isolates and Their Associations Depending on Cultivation Conditions. *AMB Express*, *9*(1), pp. 32-32.

Matthias, K.A., Roche, A.M., Standish, A.J., Shchepetov, M. and Weiser, J.N., (2008). Neutrophil-toxin interactions promote antigen delivery and mucosal clearance of Streptococcus pneumoniae. *The Journal of Immunology*, *180*(9), pp.6246-6254.

McBride, S.M. and Sonenshein, A.L., (2011). The dlt operon confers resistance to cationic antimicrobial peptides in Clostridium difficile. *Microbiology*, *157*(Pt 5), p.1457.

Meade, E., Slattery, M. A., & Garvey, M. (2020, Jan 16). Bacteriocins, Potent Antimicrobial Peptides and the Fight against Multi Drug Resistant Species: Resistance Is Futile? *Antibiotics (Basel), 9*(1), p.32.

Mellroth, P., Daniels, R., Eberhardt, A., Rönnlund, D., Blom, H., Widengren, J., Normark, S., & Henriques-Normark, B. (2012, Mar 30). LytA, major autolysin of Streptococcus pneumoniae, requires access to nascent peptidoglycan. *Journal of Biological Chemistry, 287*(14), pp.11018-11029.

Metlay, J.P., Waterer, G.W., Long, A.C., Anzueto, A., Brozek, J., Crothers, K., Cooley, L.A., Dean, N.C., Fine, M.J., Flanders, S.A. and Griffin, M.R., (2019). Diagnosis and treatment of adults with community-acquired pneumonia. An official clinical practice guideline of the American Thoracic Society and Infectious Diseases Society of America. *American journal of respiratory and critical care medicine, 200*(7), pp.e45-e67.

Miao, J., Zhou, J., Liu, G., Chen, F., Chen, Y., Gao, X., Dixon, W., Song, M., Xiao, H. and Cao, Y., (2016). Membrane disruption and DNA binding of Staphylococcus aureus cell induced by a novel antimicrobial peptide produced by Lactobacillus paracasei subsp. tolerans FX-6. *Food Control*, *59*, pp.609-613.

Miller, E.L., Abrudan, M.I., Roberts, I.S. and Rozen, D.E., (2016). Diverse ecological strategies are encoded by Streptococcus pneumoniae bacteriocin-like peptides. Genome biology and evolution, 8(4), pp.1072-1090.

Mitchell, J., (2011). Streptococcus mitis: walking the line between commensalism and pathogenesis. *Molecular oral microbiology, 26*(2), pp.89-98.

Miyaji, E.N., Oliveira, M.L.S., Carvalho, E. and Ho, P.L., (2013). Serotype-independent pneumococcal vaccines. Cellular and Molecular Life Sciences, 70(18), pp.3303-3326.

Moberley, S., Holden, J., Tatham, D.P. and Andrews, R.M., (2013). Vaccines for preventing pneumococcal infection in adults. Cochrane database of systematic reviews, (1).

Moll, G.N., Konings, W.N. and Driessen, A.J., (1999). bacteriocins: mechanism of membrane insertion and pore formation. *Antonie van Leeuwenhoek*, *76*(1), pp.185-198.

Mook-Kanamori, B.B., Geldhoff, M., van der Poll, T. and van de Beek, D., (2011). Pathogenesis and pathophysiology of pneumococcal meningitis. Clinical microbiology reviews, 24(3), pp.557-591.

Moreno-Gámez, S., Sorg, R. A., Domenech, A., Kjos, M., Weissing, F. J., van Doorn, G. S., and Veening, J.-W. (2017, 2017/10/11). Quorum Sensing Integrates Environmental Cues, Cell Density and Cell History to Control Bacterial Competence. *Nature Communications, 8*(1), pp. 854.

Morley, D., Torres, A., Cillóniz, C. and Martin-Loeches, I., (2017). Predictors of treatment failure and clinical stability in patients with community acquired pneumonia. Annals of translational medicine, 5(22), p.443.

Muñoz-Almagro, C., Ciruela, P., Esteva, C., Marco, F., Navarro, M., Bartolome, R., Sauca, G., Gallés, C., Morta, M., Ballester, F. and Raga, X., (2011). Serotypes and clones causing invasive pneumococcal disease before the use of new conjugate vaccines in Catalonia, Spain. *Journal of Infection, 63*(2), pp.151-162.

Musher, D.M. and Thorner, A.R., (2014). Community-acquired pneumonia. New England Journal of Medicine, 371(17), pp.1619-1628.

Nagano, H., Kawabata, M., Sugita, G., Tsuruhara, A., Ohori, J., Jimura, T., Miyashita, K., Kurono, Y., Tomonaga, K., Briles, D.E. and Fujihashi, K., (2018). Transcutaneous immunization with pneumococcal surface protein A in mice. The Laryngoscope, 128(3), pp.E91-E96.

Naghmouchi, K., Le Lay, C., Baah, J. and Drider, D., (2012). Antibiotic and antimicrobial peptide combinations: synergistic inhibition of Pseudomonas fluorescens and antibiotic-resistant variants. Research in microbiology, 163(2), pp.101-108.

Neef, J., Andisi, V. F., Kim, K. S., Kuipers, O. P., & Bijlsma, J. J. (2011). Deletion of a cation transporter promotes lysis in Streptococcus pneumoniae. *Infection and immunity*, *79*(6), 2314-2323.

Nelson, A.L., Roche, A.M., Gould, J.M., Chim, K., Ratner, A.J. and Weiser, J.N., (2007). Capsule enhances pneumococcal colonization by limiting mucus-mediated clearance. Infection and immunity, 75(1), pp.83-90.

Nissen-Meyer, J., Rogne, P., Oppegård, C., Haugen, H. S., and Kristiansen, P. E. (2009, Jan). Structure-Function Relationships of the Non-Lanthionine-Containing Peptide (Class Ii) bacteriocins Produced by Gram-Positive Bacteria. *Curr Pharm Biotechnol*, *10*(1), pp. 19-37.

Noda, M., Miyauchi, R., Danshiitsoodol, N., Matoba, Y., Kumagai, T., and Sugiyama, M. (2018, Apr 1). Expression of Genes Involved in bacteriocin Production and Self-Resistance in Lactobacillus Brevis 174a Is Mediated by Two Regulatory Proteins. *Appl Environ Microbiol*, *84*(7).

Nordström, K., and Dasgupta, S. (2006, 2006/05/01). Copy-Number Control of the Escherichia Coli Chromosome: A Plasmidologist's View [https://doi.org/10.1038/sj.embor.7400681]. *EMBO reports*, *7*(5), pp. 484-489.

Odell, I. D., and Cook, D. (2013, Jan). Immunofluorescence Techniques. *J Invest Dermatol*, *133*(1), pp. e4.

Ogunniyi, A. D., Mahdi, L. K., Jennings, M. P., McEwan, A. G., McDevitt, C. A., Van der Hoek, M. B.,... & Paton, J. C. (2010). Central Role of Manganese in Regulation of

Stress Responses, Physiology, and Metabolism in *Streptococcus pneumoniae*. *Journal of Bacteriology*, *192*(17), 4489-4497.

Olarte, L., Barson, W.J., Barson, R.M., Romero, J.R., Bradley, J.S., Tan, T.Q., Givner, L.B., Hoffman, J.A., Lin, P.L., Hultén, K.G. and Mason, E.O., (2017). Pneumococcal pneumonia requiring hospitalization in US children in the 13-valent pneumococcal conjugate vaccine era. Clinical Infectious Diseases, 64(12), pp.1699-1704.

Olivares-Trejo, J.de J. and Alvarez-Sánchez, M.E. (2022) *Proteins of streptococcus pneumoniae involved in iron acquisition*, *IntechOpen*. IntechOpen.

Oliver Melissa, B., Basu Roy, A., Kumar, R., Lefkowitz Elliot, J., Swords, W. E., and D'Orazio Sarah, E. F. (2017). Streptococcus Pneumoniae Tigr4 Phase-Locked Opacity Variants Differ in Virulence Phenotypes. *mSphere*, *2*(6), pp. e00386-00317.

Oliver, M. B., van der Linden, M. P. G., Küntzel, S. A., Saad, J. S., and Nahm, M. H. (2013). Discovery of Streptococcus Pneumoniae Serotype 6 Variants with Glycosyltransferases Synthesizing Two Differing Repeating Units. *The Journal of biological chemistry*, *288*(36), pp. 25976-25985.

Orange, J.S., Ballow, M., Stiehm, E.R., Ballas, Z.K., Chinen, J., De La Morena, M., Kumararatne, D., Harville, T.O., Hesterberg, P., Koleilat, M. and McGhee, S., (2012). Use and interpretation of diagnostic vaccination in primary immunodeficiency: a working group report of the Basic and Clinical Immunology Interest Section of the American Academy of Allergy, Asthma & Immunology. Journal of Allergy and Clinical Immunology, 130(3), pp.S1-S24.

Ott, S.R., Hauptmeier, B.M., Ernen, C., Lepper, P.M., Nüesch, E., Pletz, M.W., Hecht, J., Welte, T. and Bauer, T.T., (2012). Treatment failure in pneumonia: impact of antibiotic treatment and cost analysis. *European respiratory journal*, *39*(3), pp.611-618.

Pablo, M.A., Gaforio, J.J., Gallego, A.M., Ortega, E., Gálvez, A.M. and Alvarez de Cienfuegos López, G., (1999). Evaluation of immunomodulatory effects of nisin-containing diets on mice. *FEMS Immunology & Medical Microbiology*, *24*(1), pp.35-42.

Papaleo, S., Alvaro, A., Nodari, R., Panelli, S., Bitar, I., & Comandatore, F. (2022, 2022-September-15). The red thread between methylation and mutation in bacterial antibiotic resistance: How third-generation sequencing can help to unravel this relationship [Review]. *Frontiers in Microbiology*, *13*(Sep 15).

Pennington, S.H., Pojar, S., Mitsi, E., Gritzfeld, J.F., Nikolaou, E., Solórzano, C., Owugha, J.T., Masood, Q., Gordon, M.A., Wright, A.D. and Collins, A.M., (2016). Polysaccharide-specific memory B cells predict protection against experimental human pneumococcal carriage. *American journal of respiratory and critical care medicine*, *194*(12), pp.1523-1531.

Perez, R. H., Zendo, T., and Sonomoto, K. (2014, 2014/08/29). Novel bacteriocins from Lactic Acid Bacteria (Lab): Various Structures and Applications. *Microbial Cell Factories*, *13*(1), pp. S3.

Perez, R.H., Zendo, T. and Sonomoto, K., (2014). Novel bacteriocins from lactic acid bacteria (LAB): various structures and applications. Microbial cell factories, 13(1), pp.1-13.

Perez, R.H., Zendo, T. and Sonomoto, K., (2014). Novel bacteriocins from lactic acid bacteria (LAB): various structures and applications. *Microbial cell factories*, *13*(1), pp.1-13.

Pérez-Ramos, A., Madi-Moussa, D., Coucheney, F., & Drider, D. (2021, Oct 7). Current Knowledge of the Mode of Action and Immunity Mechanisms of LAB-Bacteriocins. *Microorganisms*, *9*(10).

Pernil, R., and Schleiff, E. (2019). Metalloproteins in the Biology of Heterocysts. *Life*, *9*(2), pp. 32.

Pinchas, M. D., LaCross, N. C., and Dawid, S. (2015, Apr). An Electrostatic Interaction between Blpc and Blph Dictates Pheromone Specificity in the Control of bacteriocin Production and Immunity in Streptococcus Pneumoniae. *J Bacteriol, 197*(7), pp. 1236-1248.

Pinchas, M.D., LaCross, N.C. and Dawid, S., (2015). An electrostatic interaction between BlpC and BlpH dictates pheromone specificity in the control of bacteriocin production and immunity in Streptococcus pneumoniae. Journal of bacteriology, 197(7), pp.1236-1248.

Poolman, J.T., Peeters, C.C. and Van Den Dobbelsteen, G.P., (2013). The history of pneumococcal conjugate vaccine development: dose selection. Expert review of vaccines, 12(12), pp.1379-1394.

Potter, A. J., Trappetti, C., & Paton, J. C. (2012). *Streptococcus pneumoniae* uses Glutathione to

Preciado, G.M., Michel, M.M., Villarreal-Morales, S.L., Flores-Gallegos, A.C., Aguirre-Joya, J., Morlett-Chávez, J., Aguilar, C.N. and Rodríguez-Herrera, R., (2016). Bacteriocins and its use for multidrug-resistant bacteria control. *Antibiotic Resistance: Mechanisms and New Antimicrobial Approaches*, pp.329-349.

Prince, A., Sandhu, P., Ror, P., Dash, E., Sharma, S., Arakha, M., Jha, S., Akhter, Y. and Saleem, M., (2016). Lipid-II independent antimicrobial mechanism of nisin depends on its crowding and degree of oligomerization. *Scientific reports*, *6*(1), pp.1-15.

Pumarola, F., Marès, J., Losada, I., Minguella, I., Moraga, F., Tarragó, D., Aguilera, U., Casanovas, J.M., Gadea, G., Trías, E. and Cenoz, S., 2013. Microbiology of bacteria causing recurrent acute otitis media (AOM) and AOM treatment failure in young children in Spain: shifting pathogens in the post-pneumococcal conjugate vaccination era. *International journal of pediatric otorhinolaryngology*, *77*(8), pp.1231-1236.

Pumarola, F., Marès, J., Losada, I., Minguella, I., Moraga, F., Tarragó, D., Aguilera, U., Casanovas, J.M., Gadea, G., Trías, E. and Cenoz, S., (2013). Microbiology of bacteria causing recurrent acute otitis media (AOM) and AOM treatment failure in young children in Spain: shifting pathogens in the post-pneumococcal conjugate vaccination era. *International journal of pediatric otorhinolaryngology*, *77*(8), pp.1231-1236.

Rai, P., He, F., Kwang, J., Engelward, B.P. and Chow, V.T., (2016). Pneumococcal pneumolysin induces DNA damage and cell cycle arrest. *Scientific reports*, *6*(1), pp.1-12.

Rajagopal, M., & Walker, S. (2017). Envelope structures of gram-positive bacteria. *Current Topics in Microbiology and Immunology, 404*(Jan 1), pp.1-44.

Ratner, S., Bollinger, K., Richardson, J., & Dawid, S. (2022). The outer surface protease, SepM, is required for blp locus activation in three of the four most common pherotypes of Streptococcus pneumoniae. *Journal of Bacteriology, 2022*(May 27), p.493805.

Reichmann, P., and Hakenbeck, R. (2000, Sep 15). Allelic Variation in a Peptide-Inducible Two-Component System of Streptococcus Pneumoniae. *FEMS Microbiol Lett, 190*(2), pp. 231-236.

Rezaei Javan, R., van Tonder, A. J., King, J. P., Harrold, C. L., and Brueggemann, A. B. (2018, 2018-August-27). Genome Sequencing Reveals a Large and Diverse Repertoire of Antimicrobial Peptides [Original Research]. *Frontiers in microbiology, 9*(2012).

Rezaei Javan, R., Van Tonder, A.J., King, J.P., Harrold, C.L. and Brueggemann, A.B., (2018). Genome sequencing reveals a large and diverse repertoire of antimicrobial peptides. Frontiers in Microbiology, p.2012.

Riley, M. A., and Wertz, J. E. (2002). bacteriocins: Evolution, Ecology, and Application. *Annu Rev Microbiol, 56*, pp. 117-137.

Rima, M., Rima, M., Fajloun, Z., Sabatier, J.-M., Bechinger, B., and Naas, T. (2021). Antimicrobial Peptides: A Potent Alternative to Antibiotics. *Antibiotics, 10*(9).

Ring, A., Weiser, J.N. and Tuomanen, E.I., (1998). Pneumococcal trafficking across the blood-brain barrier. Molecular analysis of a novel bidirectional pathway. *The Journal of clinical investigation*, *102*(2), pp.347-360.

Roche, A.M., Richard, A.L., Rahkola, J.T., Janoff, E.N. and Weiser, J.N., (2015). Antibody blocks acquisition of bacterial colonization through agglutination. *Mucosal immunology*, *8*(1), pp.176-185.

Rodrigues, F., Foster, D., Nicoli, E., Trotter, C., Vipond, B., Muir, P., Gonçalves, G., Januário, L. and Finn, A., (2013). Relationships between rhinitis symptoms, respiratory viral infections and nasopharyngeal colonization with Streptococcus pneumoniae, Haemophilus influenzae and Staphylococcus aureus in children attending daycare. *The Pediatric infectious disease journal*, *32*(3), pp.227-232.

Rosenberg, S. M. (1997, Dec). Mutation for Survival. *Curr Opin Genet Dev, 7*(6), pp. 829-834.

Ruffalo, M., Koyutürk, M., Ray, S., and LaFramboise, T. (2012, Sep 15). Accurate Estimation of Short Read Mapping Quality for Next-Generation Genome Sequencing. *Bioinformatics, 28*(18), pp. i349-i355.

Sabri, S., Steen, J. A., Bongers, M., Nielsen, L. K., and Vickers, C. E. (2013, 2013/06/24). Knock-in/Knock-out (Kiko) Vectors for Rapid Integration of Large DNA Sequences, Including Whole Metabolic Pathways, onto the Escherichia Coli Chromosome at Well-Characterised Loci. *Microbial Cell Factories, 12*(1), pp. 60.

Sadowy, E., & Hryniewicz, W. (2020, 2020/12/01). Identification of Streptococcus pneumoniae and other Mitis streptococci: importance of molecular methods. *European Journal of Clinical Microbiology & Infectious Diseases, 39*(12), pp.2247-2256.

Samhita, L., Raval, P. K., and Agashe, D. (2020). Global Mistranslation Increases Cell Survival under Stress in Escherichia Coli. *PLOS Genetics, 16*(3), pp. e1008654.

Sánchez-Romero, M. A., and Casadesús, J. (2020, Jan). The Bacterial Epigenome. *Nat Rev Microbiol, 18*(1), pp. 7-20.

Santagati, M., Scillato, M., Patanè, F., Aiello, C., and Stefani, S. (2012, Jun). bacteriocin-Producing Oral Streptococci and Inhibition of Respiratory Pathogens. *FEMS Immunol Med Microbiol, 65*(1), pp. 23-31.

Seemann, T. (2014, Jul 15). Prokka: Rapid Prokaryotic Genome Annotation. *Bioinformatics, 30*(14), pp. 2068-2069.

Seemann, T. (2021). *Snippy: Fast Bacterial Variant Calling from Ngs Reads*. https://github.com/tseemann/snippy

Shanker, E., and Federle, M. J. (2017). Quorum Sensing Regulation of Competence and bacteriocins in Streptococcus Pneumoniae and Mutans. *Genes, 8*(1), pp. 15.

Sharma, K., Kaur, S., Singh, R. and Kumar, N., (2021). Classification and mechanism of bacteriocin induced cell death: A review: bacteriocin classification and their mode action. *Journal of microbiology, biotechnology and food sciences*, *11*(3), pp.e3733-e3733.

Shenoy, A. T., Beno, S. M., Brissac, T., Bell, J. W., Novak, L., and Orihuela, C. J. (2018). Severity and Properties of Cardiac Damage Caused by Streptococcus Pneumoniae Are Strain Dependent. *PLOS ONE, 13*(9), pp. e0204032.

Sidhu, P., and Nehra, K. (2021). bacteriocins of Lactic Acid Bacteria as Potent Antimicrobial Peptides against Food Pathogens. In.

Simons, A., Alhanout, K., and Duval, R. E. (2020). bacteriocins, Antimicrobial Peptides from Bacterial Origin: Overview of Their Biology and Their Impact against Multidrug-Resistant Bacteria. *Microorganisms, 8*(5), pp. 639.

Slager, J., Kjos, M., Attaiech, L., and Veening, J. W. (2014, Apr 10). Antibiotic-Induced Replication Stress Triggers Bacterial Competence by Increasing Gene Dosage near the Origin. *Cell, 157*(2), pp. 395-406.

Son, M. R., Shchepetov, M., Adrian, P. V., Madhi, S. A., de Gouveia, L., von Gottberg, A., Klugman, K. P., Weiser, J. N., & Dawid, S. (2011). Conserved mutations in the pneumococcal bacteriocin transporter gene, blpA, result in a complex population consisting of producers and cheaters. *mBio, 2*(5), pp.e00179-e00211.

Son, M. R., Shchepetov, M., Adrian, P. V., Madhi, S. A., Gouveia, L. d., Gottberg, A. v., Klugman, K. P., Weiser, J. N., Dawid, S., and McDaniel, L. S. (2011). Conserved Mutations in the Pneumococcal bacteriocin Transporter Gene, <I>Blpa</I>, Result in a Complex Population Consisting of Producers and Cheaters. *mBio, 2*(5), pp. e00179-00111.

Son, M.R. *et al.* (2011) *Conserved Mutations in the Pneumococcal bacteriocin Transporter Gene, blpA, Result in a Complex Population Consisting of Producers and Cheaters*, *ASM Journals*. Available at: https://journals.asm.org/doi/10.1128/mBio.00179-11 (Accessed: January 11, 2023).

Song, J.Y., Moseley, M.A., Burton, R.L. and Nahm, M.H., (2013). Pneumococcal vaccine and opsonic pneumococcal antibody. Journal of Infection and Chemotherapy, 19(3), pp.412-425.

Spiess, C., Beil, A. and Ehrmann, M., (1999). A temperature-dependent switch from chaperone to protease in a widely conserved heat shock protein. *Cell, 97*(3), pp.339-347.

Steckbeck, J. D., Deslouches, B., and Montelaro, R. C. (2014, Jan). Antimicrobial Peptides: New Drugs for Bad Bugs? *Expert Opin Biol Ther, 14*(1), pp. 11-14.

Stevens, K. E., Chang, D., Zwack, E. E., and Sebert, M. E. (2011). Competence in Streptococcus Pneumoniae Is Regulated by the Rate of Ribosomal Decoding Errors. *mBio, 2*(5).

Subramanian, K., Henriques-Normark, B. and Normark, S., (2019). Emerging concepts in the pathogenesis of the Streptococcus pneumoniae: from nasopharyngeal colonizer to intracellular pathogen. *Cellular microbiology, 21*(11), p.e13077.

Subramanian, S. and Smith, D.L., (2015). bacteriocins from the rhizosphere microbiome–from an agriculture perspective. Frontiers in plant science, 6, p.909.

Suganthi, V. and Mohanasrinivasan, V., (2015). Optimization studies for enhanced bacteriocin production by Pediococcus pentosaceus KC692718 using response surface methodology. Journal of food science and technology, 52(6), pp.3773-3783.

Thornton, J. A. (2017). Increased Zinc Availability Enhances Initial Aggregation and Biofilm Formation of *Streptococcus pneumoniae*. *Frontiers in Cellular and Infection Microbiology, 7*, 233.

Tin Tin Htar, M., Stuurman, A.L., Ferreira, G., Alicino, C., Bollaerts, K., Paganino, C., Reinert, R.R., Schmitt, H.J., Trucchi, C., Vestraeten, T. and Ansaldi, F., (2017). Effectiveness of pneumococcal vaccines in preventing pneumonia in adults, a systematic review and meta-analyses of observational studies. PloS one, 12(5), p.e0177985.

Tong, H.H., McIver, M.A., Fisher, L.M. and DeMaria, T.F., (1999). Effect of lacto-N-neotetraose, asialoganglioside-GM1 and neuraminidase on adherence of otitis media-associated serotypes ofStreptococcus pneumoniaeto chinchilla tracheal epithelium. Microbial pathogenesis, 26(2), pp.111-119.

Torres, A., Cillóniz, C., Blasi, F., Chalmers, J.D., Gaillat, J., Dartois, N., Schmitt, H.J. and Welte, T., (2018). Burden of pneumococcal community-acquired pneumonia in adults across Europe: a literature review. Respiratory Medicine, 137, pp.6-13.

Torres, M.D., Pedron, C.N., Higashikuni, Y., Kramer, R.M., Cardoso, M.H., Oshiro, K.G., Franco, O.L., Silva Junior, P.I., Silva, F.D., Oliveira Junior, V.X. and Lu, T.K., (2018). Structure-function-guided exploration of the antimicrobial peptide polybia-CP identifies activity determinants and generates synthetic therapeutic candidates. *Communications biology*, *1*(1), pp.1-16.

Tuomanen, E.I., (1997). The biology of pneumococcal infection. Pediatric research, 42(3), pp.253-258.

Turner, P., Hinds, J., Turner, C., Jankhot, A., Gould, K., Bentley, S.D., Nosten, F. and Goldblatt, D., 2011. Improved detection of nasopharyngeal cocolonization by multiple pneumococcal serotypes by use of latex agglutination or molecular serotyping by microarray. *Journal of clinical microbiology*, *49*(5), pp.1784-1789.

Ulijasz, A.T., Andes, D.R., Glasner, J.D. and Weisblum, B., (2004). Regulation of iron transport in Streptococcus pneumoniae by RitR, an orphan response regulator. *Journal of bacteriology*, *186*(23), pp.8123-8136.

Valente, C., De Lencastre, H., and Sá-Leão, R. (2012, Dec). Pherotypes of Co-Colonizing Pneumococci among Portuguese Children. *Microb Drug Resist, 18*(6), pp. 550-554.

Verhagen, L.M., de Jonge, M.I., Burghout, P., Schraa, K., Spagnuolo, L., Mennens, S., Eleveld, M.J., van der Gaast-de Jongh, C.E., Zomer, A., Hermans, P.W. and Bootsma, H.J., (2014). Genome-wide identification of genes essential for the survival of Streptococcus pneumoniae in human saliva. *PloS one*, *9*(2), p.e89541.

Vestrheim, D. F., Gaustad, P., Aaberge, I. S., and Caugant, D. A. (2011, Oct). Pherotypes of Pneumococcal Strains Co-Existing in Healthy Children. *Infect Genet Evol, 11*(7), pp. 1703-1708.

Villamil, L., Figueras, A. and Novoa, B., (2003). Immunomodulatory effects of nisin in turbot (Scophthalmus maximus L.). *Fish & Shellfish Immunology*, *14*(2), pp.157-169.

Vögele, M., Bhaskara, R.M., Mulvihill, E., van Pee, K., Yildiz, Ö., Kühlbrandt, W., Müller, D.J. and Hummer, G., (2019). Membrane perforation by the pore-forming toxin pneumolysin. *Proceedings of the National Academy of Sciences*, *116*(27), pp.13352-13357.

Vollmer, W. (2015). Chapter 6 - Peptidoglycan. In Y.-W. Tang, M. Sussman, D. Liu, I. Poxton, & J. Schwartzman (Eds.), *Molecular Medical Microbiology (Second Edition)* (pp. 105-124). Academic Press.

Vollmer, W., Massidda, O. and Tomasz, A., (2019). The cell wall of Streptococcus pneumoniae. Microbiology spectrum, 7(3), pp.7-3.

von Wintersdorff, C. J. H., Penders, J., van Niekerk, J. M., Mills, N. D., Majumder, S., van Alphen, L. B., Savelkoul, P. H. M., and Wolffs, P. F. G. (2016). Dissemination of Antimicrobial Resistance in Microbial Ecosystems through Horizontal Gene Transfer. *Frontiers in microbiology*, *7*, pp. 173-173.

Voss, S., Hallström, T., Saleh, M., Burchhardt, G., Pribyl, T., Singh, B., Riesbeck, K., Zipfel, P.F. and Hammerschmidt, S., (2013). The choline-binding protein PspC of Streptococcus pneumoniae interacts with the C-terminal heparin-binding domain of vitronectin. Journal of Biological Chemistry, 288(22), pp.15614-15627.

Wachsman, M.B., Castilla, V., de Ruiz Holgado, A.P., de Torres, R.A., Sesma, F. and Coto, C.E., (2003). Enterocin CRL35 inhibits late stages of HSV-1 and HSV-2 replication in vitro. Antiviral Research, 58(1), pp.17-24.

Walter, N.D., Taylor Jr, T.H., Shay, D.K., Thompson, W.W., Brammer, L., Dowell, S.F., Moore, M.R. and Active Bacterial Core Surveillance Team, (2010). Influenza circulation and the burden of invasive pneumococcal pneumonia during a non-pandemic period in the United States. Clinical Infectious Diseases, 50(2), pp.175-183.

Wang, C. Y., Patel, N., Wholey, W.-Y., and Dawid, S. (2018). Abc Transporter Content Diversity in ≪Em≫Streptococcus Pneumoniae≪/Em≫ Impacts Competence Regulation and bacteriocin Production. *Proceedings of the National Academy of Sciences, 115*(25), pp. E5776.

Wang, C.Y., Patel, N., Wholey, W.Y. and Dawid, S., (2018). ABC transporter content diversity in Streptococcus pneumoniae impacts competence regulation and bacteriocin production. Proceedings of the National Academy of Sciences, 115(25), pp.E5776-E5785.

Wang, S., Zeng, X., Yang, Q., and Qiao, S. (2016). Antimicrobial Peptides as Potential Alternatives to Antibiotics in Food Animal Industry. *International journal of molecular sciences, 17*(5), pp. 603.

Watford, S., and Warrington, S. J. (2021). Bacterial DNA Mutations. In *Statpearls*. StatPearls Publishing Copyright © 2021, StatPearls Publishing LLC.

Weinberger, D. M., Trzciński, K., Lu, Y.-J., Bogaert, D., Brandes, A., Galagan, J., Anderson, P. W., Malley, R., and Lipsitch, M. (2009). Pneumococcal Capsular Polysaccharide Structure Predicts Serotype Prevalence. *PLOS Pathogens, 5*(6), pp. e1000476.

Weinberger, D.M., Malley, R. and Lipsitch, M., (2011). Serotype replacement in disease after pneumococcal vaccination. *The Lancet, 378*(9807), pp.1962-1973.

Weiser, J. N., Ferreira, D. M., and Paton, J. C. (2018). Streptococcus Pneumoniae: Transmission, Colonization and Invasion. *Nature reviews. Microbiology, 16*(6), pp. 355-367.

Weiss, G., and Carver, P. L. (2018, 2018/01/01/). Role of Divalent Metals in Infectious Disease Susceptibility and Outcome. *Clinical Microbiology and Infection, 24*(1), pp. 16-23.

White, P., Joshi, A., Rassam, P., Housden, N. G., Kaminska, R., Goult, J. D., Redfield, C., McCaughey, L. C., Walker, D., Mohammed, S., and Kleanthous, C. (2017).

Exploitation of an Iron Transporter for Bacterial Protein Antibiotic Import. *Proceedings of the National Academy of Sciences, 114*(45), pp. 12051.

WHO. (2012, Jul 6). Pneumococcal Vaccines Who Position Paper - 2012 - Recommendations. *Vaccine, 30*(32), pp. 4717-4718.

Wholey, W. Y., Kochan, T. J., Storck, D. N., and Dawid, S. (2016, Feb). Coordinated bacteriocin Expression and Competence in Streptococcus Pneumoniae Contributes to Genetic Adaptation through Neighbor Predation. *PLoS Pathog, 12*(2), pp. e1005413.

Wholey, W.Y., Abu-Khdeir, M., Yu, E.A., Siddiqui, S., Esimai, O. and Dawid, S., (2019). Characterization of the competitive pneumocin peptides of Streptococcus pneumoniae. Frontiers in cellular and infection microbiology, 9, p.55.

Wijayasri, S., Hillier, K., Lim, G.H., Harris, T.M., Wilson, S.E. and Deeks, S.L., (2019). The shifting epidemiology and serotype distribution of invasive pneumococcal disease in Ontario, Canada, 2007-2017. PLoS One, 14(12), p.e0226353.

Wilson, W. W., Wade, M. M., Holman, S. C., & Champlin, F. R. (2001, Jan). Status of methods for assessing bacterial cell surface charge properties based on zeta potential measurements. *Journal of Microbiological Methods, 43*(3), pp.153-164.

World Health Organization. (2013). *Ending Preventable Child Deaths from Pneumonia and Diarrhoea by 2025 - the Integrated Global Action Plan for Pneumonia and Diarrhoea (Gappd)*

https://apps.who.int/iris/bitstream/handle/10665/79200/9789241505239_eng.pdf

Yang, X., Liu, D., Liu, F., Wu, J., Zou, J., Xiao, X., Zhao, F., and Zhu, B. (2013, 2013/01/31). Htqc: A Fast Quality Control Toolkit for Illumina Sequencing Data. *BMC Bioinformatics, 14*(1), pp. 33.

Yesilkaya, H., Andisi, V. F., Andrew, P. W., & Bijlsma, J. J. (2013). *Streptococcus pneumoniae* and Reactive Oxygen Species: An unusual Approach to Living with Radicals. *Trends in microbiology, 21*(4), 187-195.

Yi, L., Luo, L., Chen, J., Sun, H., Wang, X., Yi, Y. and Lv, X., (2020). Cell wall and DNA damage of Staphylococcus aureus by bacteriocin BM1157. *LWT*, *134*, p.109842.

Yildirim, I., Little, B.A., Finkelstein, J., Lee, G., Hanage, W.P., Shea, K. and Pelton, S.I., (2017). Surveillance of pneumococcal colonization and invasive pneumococcal disease reveals shift in prevalent carriage serotypes in Massachusetts' children to relatively low invasiveness. *Vaccine*, *35*(32), pp.4002-4009.

Zacharof, M.P. and Lovitt, R.W., (2012). bacteriocins produced by lactic acid bacteria a review article. Apcbee Procedia, 2, pp.50-56.

Zafar, M. A., Hamaguchi, S., Zangari, T., Cammer, M., Weiser, J. N., and Thornton, J. A. (2017). Capsule Type and Amount Affect Shedding and Transmission of *Streptococcus Pneumoniae*. *mBio, 8*(4), pp. e00989-00917.

Zafar, M.A., Wang, Y., Hamaguchi, S. and Weiser, J.N., (2017). Host-to-host transmission of Streptococcus pneumoniae is driven by its inflammatory toxin, pneumolysin. *Cell host & microbe*, *21*(1), pp.73-83.

Zhang, J., Yang, Y., Yang, H., Bu, Y., Yi, H., Zhang, L., Han, X., and Ai, L. (2018, 2018-October-01). Purification and Partial Characterization of bacteriocin Lac-B23, a Novel bacteriocin Production by Lactobacillus Plantarum J23, Isolated from Chinese Traditional Fermented Milk [Original Research]. *Frontiers in Microbiology*, *9*(2165).

Zhang, S., Wang, B., Wan, L., and Li, L. M. (2017, 2017/07/11). Estimating Phred Scores of Illumina Base Calls by Logistic Regression and Sparse Modeling. *BMC Bioinformatics, 18*(1), pp. 335.

Zhang, Y., and Zheng, J. (2020). Bioinformatics of Metalloproteins and Metalloproteomes. *Molecules (Basel, Switzerland), 25*(15), pp. 3366.

Zhou, X., and Li, Y. (2015). Chapter 5 - Oral Mucosal Microbes. In X. Zhou & Y. Li (Eds.), *Atlas of Oral Microbiology* (pp. 95-107). Academic Press.

Zhu, L.H., Li, C., Wu, J.A., Liang, J.G. and Shi, Y.F., (2008, December). bacteriocins From Lactic Acid Bacteria Increases Tumor Necrosis Factor-α Expression in a Rat Kidney Model of Chronic Rejection. In Transplantation proceedings (Vol. 40, No. 10, pp. 3746-3747). Elsevier.

Zielnik-Jurkiewicz, B. and Bielicka, A., (2015). Antibiotic resistance of Streptococcus pneumoniae in children with acute otitis media treatment failure. *International Journal of Pediatric Otorhinolaryngology*, *79*(12), pp.2129-2133.

Zimina, M., Babich, O., Prosekov, A., Sukhikh, S., Ivanova, S., Shevchenko, M., & Noskova, S. (2020, Aug 28). Overview of global trends in classification, methods of preparation and application of bacteriocins. *Antibiotics (Basel), 9*(9).