# Bacteriophages and bacteriocins: agents for biocontrol of the fish pathogen *Streptococcus iniae*

**Thesis submitted by**
**Emily Wright BSc University of Miami**
**in March 2010**

**for the degree of Masters of Science**
**in the School of Veterinary and Biomedical Sciences,**
**James Cook University**

## STATEMENT OF ACCESS

I, Emily Wright, author of this work, understand that James Cook University will make this thesis available for use within the University Library and, via the Australian Digital Theses network, for use elsewhere.

I understand that, as an unpublished work, a thesis has significant protection under the Copyright Act and;

I do not wish to place any further restriction on access to this work.

Emily Wright July 2010

## DECLARATION

I, Emily Wright, author of this work, declare that this thesis is my own work and has not been submitted in any form for another degree or diploma at any university or other institution of tertiary education. Information derived from the published and unpublished work of others has been acknowledged in the text and a list of references is given.

Emily Wright July 2010

## DECLARATION ON ETHICS

The research presented and reported in this thesis was conducted within the guidelines for research ethics outlined in the *National Statement on Ethics Conduct in Research Involving Human (*1999*),* the *Joint NHMRC/AVCC Statement and Guidelines on Research Practice* (1997)*,* the *James Cook University Policy on Experimentation Ethics*. *Standard Practices and Guidelines* (2001), and the *James Cook University Statement and Guidelines on Research Practice* (2001). The proposed research methodology received clearance from the James Cook University Experimentation Ethics Review Committee (approval number A1266). No animal experiments were performed in this project (though initially planned and approved).

Emily Wright July 2010

## DECLARATION OF ELECTRONIC COPY

I declare that the electronic copy of this thesis provided to the James Cook University Library is, within the limits of the technology available, an accurate copy of the print thesis submitted.

Emily Wright July 2010

**ACKNOWLEDGMENTS**

Finishing this thesis has been no easy task, and it could not have been accomplished without the help of so many. Firstly, many thanks are due to my supervisor, Associate Professor Leigh Owens, who has helped me navigate the ever-changing path that was my project, encouraging me the whole way. Also to my co-supervisor, Dr. Jenny Elliman, who helped me in many ways, but especially by spending hours with me in the lab. Thanks also to the other staff members and students at the School of Veterinary and Biomedical Sciences who have shared their advice, skills and wisdom over the past few years. The Microbiology technicians, Karen, Kerryn, Helen and Louise, deserve special thanks for putting up with me and my seemingly constant questions at their doorstep.

My appreciation goes out to Kathy, Kjersti, Orachun, Rusaini, Ana, Cameron, Lisa, Safeena, Praveen, and the rest of the 'AquaPath' group, for their help in the lab, company in the office, problem-solving ideas, laughter and food! It has been excellent to be surrounded by such a happy, supportive group of researchers.

Thank you to Rick Webb for his generous time in the lab and great contributions to my project. Importantly, thank you to James Cook University for financial support of my life and research throughout the past few years. I would also like to thank the anonymous owners of the fish and prawn farms for allowing the collection of samples for my study. For editorial assistance, I would like to again thank Leigh and Jenny and also Dr. Jeff Warner.

Last, but not least, thank you to my family and friends, especially Tyler and Roxy, for giving me support, smiles and most of all a sense of normalcy during the best and the worst times of my project. This thesis is dedicated to you.

## ABSTRACT

*Streptococcus iniae* is one of the most widespread and costly pathogens in fish aquaculture. Bacteriophages (phages) and bacteriocins may be the optimal new therapeutic agents for *S. iniae* that lack many of the problems associated with current control measures. This study aims to discover and characterise phages and bacteriocins with activity against *S. iniae*.

Cross spotting assays and mitomycin C inductions were conducted to screen for prophage within the JCU library of 48 *S. iniae* isolates. Phages were induced with 150-350 ng $ml^{-1}$ mitomycin C, isolated through filtration and concentrated by ultracentrifugation. Phages were characterized by plaque assays on *S. iniae*, transmission electron microscope analysis, and gel electrophoresis of *Eco*RI digested phage DNA. Mitomycin C inductions revealed 20.83% of *S. iniae* isolates show growth curves indicative of lysogeny. Plaque assays confirmed inducible prophages in 14.58% of isolates. Each of the induced phages displayed distinctive host ranges within the *S. iniae* library. Four phages, vB_SinS-44, vB_SinS-45, vB_SinS-46 and vB_SinS-48 lysed over 78% of isolates, and were highly concentrated. Phage vB_SinS-45 was extracted at the highest concentration of $4.2 \times 10^{11}$ PFU $ml^{-1}$ following ultracentrifugation. Transmission electron microscope analysis revealed phage particles from these four samples that exhibited long, non-contractile tails and isometric heads consistent with morphology of *Siphoviridae*. Restriction digests of phage DNA revealed two distinct cutting patterns, indicating similarity between phages in bacteria isolated from different hosts and geographic regions. The genome sizes estimated from the restriction digest are 28.5 kb and 66 kb for vB_Sin-45 and vB_Sin-46, respectively. The phages isolated and characterized in this study are the first described lysogenic phages associated with *S. iniae*, and are excellent candidates for genetic modification for use in therapy, prevention or research of *S. iniae* infections in fish.

To isolate lytic phages, environmental samples were collected from fish and prawn farms in Queensland. Numerous methods to expose *S. iniae* lytic phages were trialed,

including enrichments in various sets of *S. iniae* isolates, filter-free isolation techniques to minimize damage to phages, and concentration of samples using ultracentrifugation and polyethylene glycol before and after enrichment processes. No lytic phages specific for *S. iniae* were successfully isolated in this study. Spatial and seasonal distribution of the host bacteria, as well as farm vaccination programs may have prevented the collection of phages from environmental samples. Lytic phages will likely be found on farms experiencing current outbreaks of *S. iniae*, none of which occurred during this study.

During the course of the project, a bacteriocin-like inhibitory substance (BLIS) with strong antibacterial activity against *S. iniae* was discovered. This BLIS, found to be produced by *Lactococcus lactis* ssp. *lactis*, was characterized through antagonism assays on the *S. iniae* library, plasmid curing tests, protein isolation, concentration, and SDS-PAGE analysis. The kinetics of production, effects on cell viability, heat, pH and enzyme tolerance of the BLIS were also determined. The BLIS was found to be a protein bacteriocin, termed BacL49, that is heat and pH stable (100°C for 60 min, pH 2.5-9.5), and sensitive to proteinase K, α-chymotrypsin, trypsin and papain. BacL49 active proteins were found to be 5 kDa and 54 kDa in size. The bacteriocin appeared not to be plasmid regulated. BacL49 is produced at the end of the log phase and antagonistic activity decreases following early stationary phase. The bacteriocin displays antagonism against 93.75% of *S.iniae* isolates. Though BacL49 is susceptible to trypsin, unlike most forms of nisin, sequencing of the protein needs to be completed before confirming that this bacteriocin is unique. BacL49 should be considered as a therapeutic agent against *S. iniae*, due to its heat and pH stability and bactericidal activity against the majority of *S. iniae* strains. BacL49 has the potential to be delivered to fish as a purified peptide or in the form of a live probiotic as *L. lactis* ssp. *lactis* L49.

Phage therapy remains an excellent potential tool for control of *S. iniae* in aquaculture, despite the inability of this study to isolate a lytic phage against the bacterium. Further study, *in vivo* trials, and manipulation of both the bacteriocin and phages isolated in this study could produce novel, effective biocontrol agents in the fight against *S. iniae*.

## TABLE OF CONTENTS

**ACKNOWLEDGMENTS** ..... iv
**ABSTRACT** ..... v
**TABLE OF CONTENTS** ..... vii
**LIST OF TABLES** ..... xii
**LIST OF FIGURES** ..... xiii
**ABBREVIATIONS** ..... xvii

**CHAPTER ONE**

**INTRODUCTION** ..... 1

**CHAPTER TWO**

**REVIEW OF THE LITERATURE** ..... 3

2.1 Introduction 3
2.2 History of *Streptococcus iniae* 3
2.3 Characterisation of *Streptococcus iniae* 6
2.4 Identification of *Streptococcus iniae* 6
2.5 Risk Factors for *Streptococcus iniae* Infection in Fish 7
2.6 Signs of Disease in Fish 8
2.7 Portal of Entry and Mode of Transmission of *Streptococcus iniae* 9
2.8 Virulence Factors of *Streptococcus iniae* 12
2.9 Treatment Options 15
2.9.1 Vaccinations 15
2.9.2 Antibiotics 16
2.9.3 Green solutions 17
2.9.4 Probiotics 19
2.9.5 New treatment options 19
2.10 Bacteriophages 19
2.10.1 How bacteriophages operate 20
2.10.2 Lytic bacteriophages 20
2.10.3 Lysogenic bacteriophages 20
2.10.4 Bacteriophage research 22
2.10.5 Bacteriophage therapy (lytic whole phages) 24
2.10.5.1 Improvements to phage therapy 26
2.10.5.2 Previous successes with phage therapy 28
2.10.5.3 Phage therapy in aquaculture 29
2.10.5.4 Phage therapy for *Streptococcus iniae* 30

2.10.6 Whole phage prophylaxis 31
2.10.7 Lysogenic phages in therapy 31
2.10.8 Other phage applications 32
2.11 Bacteriocins 33
2.11.1 Bacteriocin research 34
2.11.2 Characterisation of bacteriocin-like inhibitory substances 35
2.11.3 Bacteriocins in treatment of bacterial infections 37
2.11.4 Possible treatment issues 37
2.11.5 Bacteriocins and *Streptococcus iniae* 38
2.12 Conclusion 38

**CHAPTER THREE**

**GENERAL MATERIALS AND METHODS**……40

3.1 Bacteriology 40
3.1.1 Trial bacterial isolates 40
3.1.2 Preservation and recovery of bacterial isolates 42
3.1.3 Culture media 42
3.1.4 Preparation of broth cultures 42
3.1.5 Bacterial lawn technique 42
3.2 Polymerase Chain Reaction Identification of Bacterial Isolates 42
3.3 Spectrophotometric Analysis of Bacterial Cultures 43
3.4 Ultracentrifugation 43
3.5 Transmission Electron Microscopy 43
3.6 Phage Nomenclature 44
3.7 Biosafety Approval 44

**CHAPTER FOUR**

**INDUCTION OF LYSOGENIC BACTERIOPHAGES FROM *STREPTOCOCCUS INIAE***…….…….....…45

4.1 Introduction 45
4.2 Materials and Methods 47
4.2.1 Antagonism screening 47
4.2.2 Mitomycin C assays 47
4.2.3 Isolation and concentration of bacteriophages 48
4.2.4 Host range determination against *S. iniae* isolates 48
4.2.5 Determination of phage concentration 48
4.2.6 Transmission electron microscopy 49
4.2.7 Bacteriophage DNA extraction 49
4.2.8 Restriction digest assay 50

4.3 Results 50
4.3.1 Antagonism screening 50
4.3.2 Mitomycin C assays 51
4.3.3 Phage host range against *Streptococcus iniae* 52
4.3.4 Phage concentrations 54
4.3.5 Transmission electron microscope analysis 54
4.3.6 Restriction digests of phage DNA 55
4.4 Discussion 58
4.5 Conclusion 64

**CHAPTER FIVE**

**METHODS FOR ISOLATING *STREPTOCOCCUS INIAE* LYTIC PHAGES**…..65

5.1 Introduction 65
5.2 Materials and Methods 66
5.2.1 Collection of samples 66
5.2.2 Liquid medium screening 67
5.2.3 Enrichments and incubations 67
5.2.4 Enrichment in 'susceptible' isolates 68
5.2.5 Enrichment in other isolates 68
5.2.6 Reduction of mechanical damage to phages 69
5.2.7 Effect of filtration on isolation of phages 69
5.2.8 Repeated enrichments and concentration through ultracentrifugation and polyethylene glycol 70
5.2.9 Ultracentrifugation of environmental samples 70
5.2.10 Enrichment with 'naïve' isolates deduced from mitomycin C Tests 71
5.2.11 Low temperature enrichments 72
5.2.12 Control experiments 73
5.3 Results 74
5.3.1 Liquid media screening 74
5.3.2 Enrichment in 'susceptible' isolates 76
5.3.3 Reduction of mechanical damage to phages 76
5.3.4 Low temperature enrichments 76
5.3.5 Control experiments 77
5.4 Discussion 79
5.5 Conclusion 85

**CHAPTER SIX**

**CHARACTERISATION OF A BACTERIOCIN ACTIVE AGAINST *STREPTOCOCCUS INIAE***……...…86


6.1 Introduction 86
6.2 Materials and Methods 87
6.2.1 Source and identification of bacteriocin-like inhibitory substance producer 87
6.2.2 Isolation of bacteriocin-like inhibitory substance L57 from bacterial culture 88
6.2.3 Confirmation of bacteriocin-like inhibitory activity 88
6.2.4 Assays for antibacterial activity 88
6.2.4.1 Spot-on-lawn assay 88
6.2.4.2 Microtitre plate assay 89
6.2.5 Ampicillin test of microtitre assay 89
6.2.6 Activity spectrum screening 89
6.2.7 Plasmid curing of *L. lactis* L57 90
6.2.8 Effect of heat, pH and enzymes on activity 90
6.2.9 Kinetics of production 91
6.2.10 Activity 91
6.2.11 Protein purification 92
6.2.11.1 Ammonium sulphate precipitation 92
6.2.11.2 HA filtration 92
6.2.12 Activity assay of partially purified proteins 93
6.2.13 Electrophoresis of partially purified proteins 93
6.3 Results 94
6.3.1 Identification of bacteriocin-like inhibitory substance producer 94
6.3.2 Confirmation of bacteriocin-like inhibitory activity 94
6.3.3 Activity Spectrum of *L. lactis* L57 96
6.3.4 Ampicillin test 98
6.3.5 Plasmid curing of *L. lactis* L57 98
6.3.6 Effect of heat, pH and enzymes on activity 99
6.3.7 Kinetics of production 101
6.3.8 Activity 101
6.3.9 Protein concentration 103
6.3.10 Protein electrophoresis 103
6.4 Discussion 109
6.5 Conclusion 113

**CHAPTER SEVEN**

**GENERAL DISCUSSION**…….….115

**LITERATURE CITED**…….…...119

**APPENDIX 1**

**BUFFERS, SOLUTIONS AND AGARS**……...134

1.1 Tris HCl (1M) 134
1.2 EDTA 134
1.3 TE Buffer 134
1.4 CSOA Selective agar 134
1.5 Phosphate Buffered Saline (PBS) X 10 134
1.6 SM buffer 135
1.7 2% gelatine 135
1.8 Ammonium Sulphate solution (saturated) 135
1.9 Destaining solution (10% acetic acid) 135
1.10 Protein Elution Buffer 135
1.11 Sodium Dodecyl Sulphate (SDS) (20%) 135

**APPENDIX 2**

**POLYMERASE CHAIN REACTION PRIMERS AND REACTION CONDITIONS**…….....136

**APPENDIX 3**

**ENVIRONMENTAL SAMPLES** …….…..137

## LIST OF TABLES


| | | |
|---|---|---|
| **Table 3.1** | Trial bacterial isolates | 41 |
| **Table 4.1** | Direct antagonistic activity produced by *S. iniae* supernatants against other *S. iniae* host isolates. | 51 |
| **Table 4.2** | Plaque forming unit measurements of concentrated induced phage samples from *S. iniae*. | 54 |
| **Table 4.3** | Measurements of phage particles derived from TEM. Average dimensions were based on a minimum of four phages. | 55 |
| **Table 4.4** | Band sizes of *Eco*RI digested lysogenic phage genomic DNA derived from GeneTools analysis. | 57 |
| **Table 5.1** | Average growth percentages of each bacterial isolate when treated with Farm B environmental samples in the liquid media screen. | 75 |
| **Table 5.2** | Characteristics of pathogenic aquatic bacteria and associated lytic phages. | 84 |
| **Table 6.1** | Antagonistic activity by BLIS-L57 from *L. lactis* L57 against indicator *S. iniae* S23 following different treatments. | 99 |

## LIST OF FIGURES


**Figure 4.1** Growth curves indicative of lysogeny measured from *S. iniae* S48, showing a steep decline in optical density of the treated culture between 3-7 hours after the addition of mitomycin C. 51

**Figure 4.2** Growth curve indicative of a non-lysogenic strain measured from *S. iniae* S12 52

**Figure 4.3** Bacterial lawn of *S. iniae* S23 spotted with concentrated induced samples from *S. iniae* isolates S3, S14, S19, S44, S45, S46 and S48. The induced sample from S3 produced no lysis on S23, exhibiting the same spot appearance as the control (not shown). Individual plaques can be seen from phages vB_Sin-14 and vB_Sin-19 and confluent lysis was produced by phages vB_Sin-44, vB_Sin-45, vB_Sin-46 and vB_Sin-48 on this host. 53

**Figure 4.4** Percentage of *S. iniae* isolates lysed by induced *S. iniae* lysogenic phages. 53

**Figure 4.5** TEM photos of lysogenic phages induced from *S. iniae*. A: vB_Sin-44; B: vB_Sin-45 with visible base plate (arrow); C: vB_Sin-46; D: vB_Sin-48. The scale bars represent 100nm. 55

**Figure 4.6** *Eco*RI restriction digest of lysogenic phage genomic DNA. Lanes 1,9: molecular weight standards; lane 2: vB_Sin-44; lane 3: vB_Sin-45; lane 4: vB_Sin-46; lane 5: vB_Sin-48; lane 6: vB_Sin-3; lane 7: vB_Sin-14; lane 8: vB_Sin-19. 57

**Figure 5.1** Optical densities of *S. iniae* strain S47 when combined with environmental samples from Farm B. 74

**Figure 5.2** TEM photos of Special Phage Holdings phages from sample SPH-2 enriched with *S. iniae*. 78

**Figure 5.3** Plaques (examples shown by arrows) caused by enriched Special Phage Holdings phages on *S. iniae* S3. 78

**Figure 6.1** Preliminary antagonistic tests with *L. lactis* L57. A: L57 (bottom) and cfs (top) spotted directly on *S. iniae* S56 lawn. B: L57 (bottom) and cfs (top) spotted on flip plate of S56 lawn. 95

**Figure 6.2** *L. lactis* L57 spotted onto indicator lawns of *S. iniae* S23 and grown aerobically (A) and anaerobically (B). 95

**Figure 6.3** Deferred antagonism assay showing all bacterial isolates from the collection streaked away from *L. lactis* L57. A: Strains 1-20 with *L. lactis* L57 (1-11 on right, 12-20 on left). B: Strains 21-40 with *L. lactis* L57 (21-30 on right, 31-40 on left). C: Strains 41-57 with *L. lactis* L57 (41-50 on right, 51-57 on left). 96

**Figure 6.4** Zones of inhibition of bacterial strains produced by *L. lactis* 57 in the deferred antagonism assay. Isolate prefixes: (S) *Streptococcus iniae*, (C) *Carnobacterium divergens*, (E) *Enterococcus faecalis*, (Cf) *Citrobacter freundii*, (L) *Lactococcus lactis* ssp. *lactis*. 97

**Figure 6.5** Optical density measurements of *S. iniae* S23 after additions of ampicillin at the indicated times. 98

**Figure 6.6** Spot-on-lawn assays for heat (A), pH (B), and enzyme (C) effects on the activity of BLIS-L57. Soft top lawns seeded with *S. iniae* S23 were used as indicators. 100

**Figure 6.7** Kinetics of BLIS-L57 production during the growth of *L. lactis* 57. The growth curve of the bacteria was generated by measurements of $OD_{600}$ and BLIS-L57 production was evaluated by measuring the clearance produced on indicator *S. iniae* S23 by cfs samples collected throughout the period shown. 101

**Figure 6.8** Mode of action of BLIS-L57 on the growth of *S. iniae* S23. The BLIS-L57 cfs (treatment cultures) or the HIB supernatant (control) was added directly after the zero minute reading (during early exponential growth phase). 102

**Figure 6.9** Effects of BLIS-L57 on the cell viability of *S. iniae* S23. The BLIS-L57 cfs (treatment culture) or the HIB supernatant (control) was added during mid log-growth phase. 102

**Figure 6.10** Tris-glycine SDS gel showing protein separations from different concentrations of BLIS-L57 produced by *L. lactis* L57. Lane 1: untreated cfs; lane 2: primary ammonium sulphate precipitate; lane 3: secondary ammonium sulphate precipitate; lane 4: HA derived proteins; lane 5: HA filtrate; lane 6: protein standards. 104

**Figure 6.11** A: Tris-glycine SDS gel showing triplicate protein separations of ammonium sulphate concentrated BLIS-L57 from *L. lactis* L57. Lanes 1, 3: secondary ammonium sulphate precipitated proteins; lane 5: protein standards. B: Spot-on-lawn antagonism assay on *S. iniae* S23 for proteins eluted from tris-glycine SDS gel run of concentrated BLIS-L57 from *L. lactis* L57. 106

**Figure 6.12** Silver stained tris-tricine SDS-PAGE showing protein separations of ammonium sulphate and HA concentrated BLIS-L57 from *L. lactis* L57. Lane 1: Protein standards; lane 2: secondary 107

ammonium sulphate precipitate; lane 3: primary ammonium sulphate precipitate; lane 4: HA derived proteins.

**Figure 6.13** A: Tris-tricine SDS gel showing protein separations of ammonium sulphate and HA concentrated BLIS-L57 from *L. lactis* L57. Lane 1: HA derived proteins; lane 2: primary ammonium sulphate precipitate; lanes 3, 4: secondary ammonium sulphate precipitate; lane 5: protein standards. B: Spot-on-lawn antagonism assay on *S. iniae* S23 for proteins eluted from tris-tricine SDS gel run of concentrated BLIS-L57 from *L. lactis* L57. 108

## ABBREVIATIONS

| | |
|---|---|
| ADH | arginine dehydrolase |
| ATP | adenosine triphosphate |
| AU | arbitrary units |
| BA | blood agar |
| BLIS | bacteriocin-like inhibitory substance |
| cfs | cell-free supernatant |
| CFU | cell forming units |
| CNS | central nervous system |
| CSOA | colistin sulphate and oxolinic acid |
| DNA | deoxyribonucleic acid |
| ECP | extracellular products |
| EDTA | ethylenediaminetetraacetic acid |
| FDA | Food and Drug Administration |
| FKC | formalin-killed cells |
| $H_2O$ | water |
| HCl | hydrochloric acid |
| HIA | heart infusion agar |
| HIB | heart infusion broth |
| IFAT | indirect fluorescent antibody technique |
| JCU | James Cook University |
| MMC | mitomycin C |
| $OD_{540}$ | optical density at 540 nm |
| $OD_{600}$ | optical density at 600 nm |
| $OD_{260/280}$ | ratio of optical density at 260 nm and 280 nm |
| PBS | phosphate buffered saline |
| PCR | polymerase chain reaction |
| PEG | polyethylene glycol |
| PFGE | pulsed-field gel electrophoresis |
| PFU | plaque forming units |

| | |
|---|---|
| PGM | phosphoglucomutase |
| QLD DPI | Queensland Department of Primary Industries |
| RNA | ribonucleic acid |
| rRNA | ribosomal ribonucleic acid |
| SDS | sodium dodecyl suphate |
| SDS-PAGE | sodium dodecyl suphate polyacrilamide gel electrophoresis |
| SLS | Streptolysin S |
| SPH | Special Phage Holdings |
| TEM | transmission electron microscope |
| TE | tris-EDTA |
| VLP | virus-like particles |
| WA DA | Western Australia Department of Agriculture |

# CHAPTER ONE

## GENERAL INTRODUCTION

Streptococcosis is currently one of the largest disease problems facing aquaculture worldwide. Streptococcal infections affect a wide variety of wild and farmed fish and are responsible for increasing economic losses in fish production each year (Bercovier *et al.*, 1997; Austin and Austin, 1999). There are multiple Gram-positive bacteria known to cause streptococcosis, including *Streptococcus parauberis*, *Streptococcus iniae*, *Streptococcus difficilis*, *Lactococcus garvieae*, *Lactococcus piscium*, *Vagococcus salmoninarum*, and *Carnobacterium piscicola* (Eldar *et al.*, 1997a; Eldar and Ghittino, 1999; Mata *et al.*, 2004b). *Streptococcus iniae* is one of the most widespread and economically important of these pathogens (Creeper and Buller, 2006). *S. iniae* affects many species of fish worldwide, and can also cause systemic disease in humans (Austin and Austin, 1999; Lau *et al.*, 2003).

Research to discover virulence factors and transmission routes for *S. iniae* is ongoing, but prevention and treatment of this bacterium in aquaculture remains difficult. Vaccinations and antibiotics have been effective at reducing mortality due to *S. iniae* infections in commercially grown fish, but regular use of these treatments has led to bacterial resistance and the evolution of new *S. iniae* strains that are even more pathogenic (Bachrach *et al.*, 2001). Several chemical-free solutions have been proposed for the treatment of *S. iniae* infection, but none yet have proved efficient at preventing high levels of mortality from the disease. Novel antimicrobial solutions are needed to fight this pathogen.

Bacteriophages (phages) and bacteriocins could be optimal new therapeutic agents for streptococcosis caused by *S. iniae*. As such, they are the basis of my investigations. Phages are viruses that kill bacteria and are naturally abundant, have specific host ranges and can replicate quickly in the presence of host cells. Whole phage therapy has proven

effective against multi-drug resistant bacteria (Skurnik *et al.*, 2007), and has been successful at treating bacterial pathogens in humans, other mammals and cultured fish (Alisky *et al.*, 1998; Bull *et al.*, 2002; Park and Nakai, 2003). Bacteriocins are biologically active proteins or protein complexes displaying a bactericidal mode of action towards closely related species (Tagg and McGiven, 1971). These antimicrobial peptides are recognized as potentially useful agents in the control of bacterial infections due to their effectiveness, non-toxicity and relatively cheap production (Brook, 1999; Reid *et al.*, 2001; Riley and Wertz, 2002). Phages and bacteriocins are receiving growing attention as alternative treatments for bacterial infections, and my research aims to find and characterise both of these agents specifically for the treatment and prevention of *S. iniae* in fish.

# CHAPTER TWO

# REVIEW OF THE LITERATURE

## 2.1 Introduction

Due to the economic importance and zoonotic potential of *S. iniae*, this pathogen has been the subject of a substantial amount of research in recent decades. The following is a review of current knowledge concerning infections caused by *S. iniae*, virulence factors of the bacterium, and most importantly, the treatment and prevention options available for *S. iniae*. In the latter part of the review, bacteriophages and bacteriocins will be introduced and discussed in terms of their potential for fighting bacterial pathogens in aquaculture.

## 2.2 History of *Streptococcus iniae*

*S. iniae* was first reported from an Amazonian freshwater dolphin (*Inia geoffrensis*) with "golf ball disease" in San Francisco (Pier and Madin, 1976). The bacterium was later discovered in another freshwater dolphin in an aquarium in New York (Pier *et al.*, 1978), and in a third in 1987 (Bonar and Wagner, 2003). Several outbreaks of *S. iniae* occurred in fish during the 1970's and 1980's in Japan (Kitao *et al.*, 1981; Nguyen *et al.*, 2002), Singapore (Foo *et al.*, 1985; Stoffregen *et al.*, 1996), Israel and Taiwan (Eldar *et al.*, 1994), but most of these infections were initially misdiagnosed as other bacteria, and were only later recognized to be caused by *S. iniae*. In 1994 and 1995, *S. iniae* was cultured from rainbow trout (*Oncorhynchus mykiss*) and tilapia (*Oreochromis* spp.) in Israel (Eldar *et al.*, 1995), in tilapia and striped bass (*Morone saxitalis*) in the United States (Perera *et al.*, 1997), and also in barramundi (*Lates calcarifer*) in Australia (Bromage *et al.*, 1999). In late 1999, a massive fish kill due to *S. iniae* occurred in the south-east Caribbean basin, affecting a wide range of reef fish species, and proving that this pathogen is present in wild fish (Ferguson *et al.*, 2000). *S. iniae* has also been isolated from cultured bullfrogs (*Rana castesbeiana*) (Mauel *et al.*, 2002).

*S. iniae* is widely distributed, causing severe damage to fisheries in the United States, Australia, Europe, Israel, Japan, Saudi Arabia, and South Africa, as reviewed in Austin and Austin (1999). Aquaculture industries in many Asian countries including Korea, China and Japan also experience acute mortality due to *S. iniae* (Stoffregen *et al.*, 1996; Nguyen *et al.*, 2002; Nho *et al.*, 2009). Fish losses due to *S. iniae* can be severe, sometimes exceeding over half of the population of infected stocks (Bromage *et al.*, 1999). *S. iniae* epizootics cause heavy economic losses estimated to be over $150 million worldwide (Shoemaker *et al.*, 2000; Buchanan *et al.*, 2005). *S. iniae* causes disease and mortality in at least 30 species of cultured and wild fish in marine, brackish and freshwater environments (Buchanan *et al.*, 2005; Creeper and Buller, 2006). Some susceptible species include hybrid striped bass *Morone chrysops* x *Morone saxitalis* (Stoffregen *et al.*, 1996), rainbow trout *Oncorhynchus mykiss* (Eldar *et al.*, 1995), tilapia *Oreochromis* sp. (Evans *et al.*, 2006), barramundi *Lates calcarifer* (Bromage and Owens, 2002; Nawawi *et al.*, 2009), flounder *Paralichthys olivaceus* (Nho *et al.*, 2009), and sardines *Sardinops melanostictus* (Kasuda and Salati, 1999). Different species of fish appear to be more susceptible to *S. iniae* infection than others (Zlotkin *et al.*, 1998; Evans *et al.*, 2000).

In Australia, *S. iniae* infections have been confirmed in Queensland (Bromage *et al.*, 1999), and more recently, in Western Australia, South Australia and the Northern Territory (Creeper and Buller, 2006). In 2003, a barramundi cage-culture farm in Western Australia experienced over 35% mortality during a 30-day outbreak of *S. iniae* that cost AUD$2 million (Creeper and Buller, 2006). Another barramundi farm in northern Queensland lost an estimated 60 tonnes of fish due to *S. iniae* in March of 2007, which cost the farm over AUD$300,000 (Anon, 2007).

Disease-causing and commensal strains of *S. iniae* exist (Fuller *et al.*, 2002), though research has focused heavily on the virulent strains due to their economic importance. Differences between geographically disparate strains of *S. iniae* have been identified. Pathogenic strains producing a similar disease process were differentiated between North America and Israel by ribotyping (Eldar *et al.*, 1997b). Pulsed-field gel

electrophoresis (PFGE) was used to differentiate clinical isolates of *S. iniae* from Hong Kong and Canada (Lau *et al.*, 2006). Phenotypic differences were also observed: Asian isolates were larger, more β-haemolytic and mucoid than those from North America. North American clinical isolates were found to be very similar to fish isolates from Australia when analysed by PFGE (Nawawi *et al.*, 2008). Also, the dolphin-isolated type strains have been found to group separately from strains isolated from fish and humans (Eldar *et al.*, 1997b; Weinstein *et al.*, 1997; Facklam *et al.*, 2005; Nawawi *et al.*, 2008).

Regular vaccination of fish in Israel from 1995 to 1997 appears to have caused the evolution of a second serotype of *S. iniae*. The early (pre-Israel vaccinations) and old (post-Israel vaccinations) types of *S. iniae* were designated serotype I and serotype II, respectively (Bachrach *et al.*, 2001). A more recent, host-directed evolution of the bacteria has also been detected in Australia, though it is likely not linked to changes in virulence. Strains of *S. iniae* isolated from a single marine barramundi farm have evolved a larger lactate oxidase gene (*lctO*) than other strains isolated around the world, allowing them to process lactate at a faster rate (Nawawi *et al.*, 2009).

*S. iniae* can also cause invasive disease in humans, leading to cellulitis, meningitis, bacteraemias, and cutaneous and systemic pyogenic infections (Weinstein *et al.*, 1997). At least 28 cases of human infection have been reported from the United States, Canada, Hong Kong, Singapore and Taiwan, but the number of actual cases may be underestimated due to the difficulty in identifying *S. iniae* (Lau *et al.*, 2003; Facklam *et al.*, 2005; Lau *et al.*, 2006; Sun *et al.*, 2007). Most people who become infected with *S. iniae* contract the bacterium while preparing whole, fresh fish (mainly tilapia), and most patients are older (mean age of 70), of Asian descent, and have pre-existing medical conditions (Lau *et al.*, 2003; Sun *et al.*, 2007).

## 2.3 Characterisation of *Streptococcus iniae*

*Streptococcus iniae* is a Gram-positive bacterium, with cocci measuring 0.3 × 0.5 μm that appear in clusters and chains and have diplococcus formation (Kasuda and Salati, 1999; Roberts, 2001; Bolotin *et al.*, 2007). *S. iniae* is non-motile, non-spore forming, and is a facultative anaerobe. Although the bacterium is considered to be β-haemolytic, α-haemolytic strains are not uncommon, as well as the occasional strain showing a double zone of haemolysis (Ferguson *et al.*, 2000; Colorni *et al.*, 2002). The bacterium is catalase negative, and can ferment glucose, maltose, mannitol, mannose, salicin, sucrose and trehalose. *S. iniae* serotype I strains are arginine dehydrolase (ADH) positive, while serotype II strains are ADH negative (Barnes *et al.*, 2003b).

*S. iniae* shows temperature dependence; it normally appears in hosts during warm seasons or in warm waters (Bromage *et al.*, 1999; Kasuda and Salati, 1999). The bacterium has a short incubation period and can exist freely in the environment (Bromage and Owens, 2002). *S. iniae* can remain viable up to 5 days in freshwater after washing, and even longer in saline mixtures (Perera *et al.*, 1997), suggesting that the bacteria could remain viable in the environment at low densities during cooler seasons.

## 2.4 Identification of *Streptococcus iniae*

*S. iniae* is difficult to identify; it does not react to Lancefield A-V grouping antisera (Shoemaker *et al.*, 2000), and no commercial bacterial identification system includes *S. iniae* in its database (Mata *et al.*, 2004a; Roach *et al.*, 2006). The difficulty in accurately identifying *S. iniae* with commercial identification systems means that *S. iniae* is easily misidentified and could have been responsible for more fish epizootics (and also human infections) in the past several decades than reported. Several systems and techniques for the identification of *S. iniae* have been developed in recent years.

The Biolog system (Biolog Microlog® Bacterial Identification System) is able to identify approximately 70% of *S. iniae* strains (Roach *et al.*, 2006). With this efficiency,

Biolog needs to be used in conjunction with epidemiological data, clinical findings and specific PCR techniques to accurately identify *S. iniae* (Roach *et al.*, 2006). The least time consuming and most efficient system for identifying *S. iniae* is currently the indirect fluorescent antibody technique (IFAT), which employs a specific monoclonal antibody for *S. iniae*. IFAT can reliably detect the bacterium from nare samples of infected fish within 24 hours of mortality and also from nares of live asymptomatic carriers (Klesius *et al.*, 2006).

PCR (polymerase chain reaction) is currently the most reliable method of identification for *S. iniae*. There are *S. iniae* primers in the 16S to 23S ribosomal DNA intergenic spacer (Berridge *et al.*, 1998), and from the 16S ribosomal RNA gene sequence (Zlotkin *et al.*, 1998). A more useful PCR assay for rapid and accurate diagnoses of *S. iniae* is based on the lactate oxidase (*lctO*) gene (Mata *et al.*, 2004a). The latter assay is more reliable due to nonspecific amplification of *Streptococcus difficilis* that was reported with the 16S rRNA assay (Mata *et al.*, 2004a). A multiplex PCR assay has been developed to detect *S. iniae* along with other pathogens most commonly responsible for causing streptococcosis in warm-water fish, *S. difficilis*, *S. parauberis* and *L. garvieae* (Mata *et al.*, 2004b).

### 2.5 Risk Factors for *Streptococcus iniae* Infection in Fish

Like many other diseases of marine and aquatic animals, the density of the host population has a significant effect on mortality caused by *S. iniae*. Tilapia infected by immersion in *S. iniae* experienced significantly higher mortality when living at concentrations ≥ 11.2 g/l, even with half water exchanges being performed every hour (Shoemaker *et al.*, 2000). This raises a flag for susceptibility of fish in aquaculture, as most recirculating production facilities keep fish at densities much higher than this (30-290 g/l in commercial tilapia farms in the United States) with little or no exchange of water (Shoemaker *et al.*, 2000). Besides overstocking, $H_2S$ generation by vegetation around animal enclosures can cause stressed fish to swim to deeper, colder (hypoxic) waters, increasing stress and density of the fish (Creeper and Buller, 2006). Low oxygen levels alone were not found to influence mortality from *S. iniae* infection (Bowser *et al.*,

1998). Due to the temperature dependence of *S. iniae*, outbreaks in fish stocks are more likely to occur when water temperatures are above 17°C in cold water species (Bercovier *et al.*, 1997), and between 25 and 28°C in warm water species (Bromage and Owens, 2009). When conditions are right for the bacterium, small concentrations are capable of causing infection in fish. *S. iniae* concentrations as low as $1.0 \times 10^3$ to $3.2 \times 10^4$ cfu/fish can constitute a lethal dose for barramundi when contracted orally (Bromage and Owens, 2002).

## 2.6 Signs of Disease in Fish

Central nervous system (CNS) malfunction is the main clinical sign of infection with both serotypes of *S. iniae* (Lahav *et al.*, 2004). CNS malfunction can lead to spinning or eratic swimming patterns, dorsal rigidity and lethargy (Austin and Austin, 1999). Fish infected with serotype II often exhibit signs of anorexia, darkening of the skin, multifocal ecchymoses, gill pallor, and very infrequently, inflammation of the anus (Lahav *et al.*, 2004). Infected fish can display pronounced congestion and haemorrhages, especially at the base of the pectoral fins and over the heart. Exopthalmia, panophthalmus, corneal opacity, intraocular and periorbital haemorrhages are all important signs of *S. iniae* localization in the eye (Lahav *et al.*, 2004). These signs of high bacterial concentrations in the eye normally occur shortly before death, suggesting that the eye may serve as the last target organ in the disease process (Evans *et al.*, 2001).

The highest bacterial loads found in fish infected with both serotypes of *S. iniae* are in the central nervous system, though there are normally more bacteria present in the brains of fish infected with serotype II (Lahav *et al.*, 2004). *S. iniae* can cause fatal meningoencephalitis in the brain and lesions in the nervous system, which lead to the loss of orientation and lethargy observed in infected fish (Lahav *et al.*, 2004). Infected fish may show a severe inflammatory response with high levels of infiltration of neutrophils and macrophages, though this is more common with serotype II.
The most common internal change in *S. iniae* infected fish is splenomegaly (Lahav *et al.*, 2004). Infected fish also often display intravascular lesions and congestion at the

fins and mouth. The disease can lead to embolic infarction, often with focal necrosis and congestion in the posterior kidney, spleen and liver (Roberts, 2001; Buchanan *et al.*, 2005). The disease is septicaemic and frequently chronic, causing a pale colored liver and deep red spleen (Stoskopf, 1993; Kasuda and Salati, 1999). Internally, *S. iniae* serotype II causes a systemic disease with diffuse necrotizing myositis, internal lesions and haemorrhaging, especially in the spleen and fat surrounding the intestine. Somatic muscles can show multifocal to diffuse degeneration and necrosis (Lahav *et al.*, 2004). Different species of fish may react differently to *S. iniae* depending on the serotype, strain and route of infection.

Acute and subacute forms of *S. iniae* infection occur in euryhaline barramundi (Bromage and Owens, 2002). Barramundi undergoing an acute infection (inoculated by intraperitoneal injection or immersion) normally die within 24-48 hours of infection. These fish show limited signs of disease except for occasional mild corneal opacity. Mortality of co-habitating fish is the only reliable sign of acute infection as other fish generally experience more rapid mortality than barramundi. Barramundi experiencing a subacute infection (exposed orally) show displays of exopthalmia and darkened coloration, and swim spirally near the surface of the water. Subacute infection may cause only 1% mortality. With either form of infection, *S. iniae* can be isolated primarily from the brain and spleen of the fish (Bromage, 1997). Fish can also be subclinically infected with *S. iniae*, carrying the disease in the brain asymptomatically (Bromage *et al.*, 1999). Stress may induce symptoms in subclinically infected fish, but this level of infection can be hard to confirm with standard tests (Evans *et al.*, 2006).

### 2.7 Portal of Entry and Mode of Transmission of *Streptococcus iniae*

Research to discover the portal of entry of *S. iniae* to host fish is ongoing, and many suggestions have been made. One popular idea is that fish are infected orally by contaminated food (Shoemaker *et al.*, 2000; Seng and Colorni, 2002). Careful handling and protection of food from contamination have reduced infections in many farms (Bromage and Owens, 2002). However, a recent study from Korea showed that trash fish served as food to cultured flounder were contaminated with *S. iniae* and other

pathogens (Kim *et al.*, 2007a). Alternate portals of entry tested in hybrid striped bass are the gills and nares. *S. iniae* can enter the fish through the gills (McNulty *et al.*, 2003), but this infection route causes lower mortality than inoculation of the nares with similar concentrations of bacteria (Evans *et al.*, 2000). The nares appear to be a common route of entry for *S. iniae* to cultured fish (Evans *et al.*, 2000; Shoemaker *et al.*, 2000).

Cannibalism could be a mode of disease transmission through fish stocks (Shoemaker *et al.*, 2000). Healthy tilapia were observed to cannibalize dead tilapia infected with *S. iniae* that were left in their environment for just 48 hours, which then lead to *S. iniae* induced mortality in these fish (Shoemaker *et al.*, 2000). This study shows the importance of removing dead fish from the environment as quickly as possible to prevent transmission by this route. It may not be possible to eliminate *S. iniae* from surviving fish after an outbreak, leaving open the possibility of re-infection (Austin and Austin, 1999). In the recent *S. iniae* outbreak in Western Australia, carrier fish must have survived and later shed the bacteria while stressed because there were mortality spikes during the following months after stressful events such as algal blooms and water inversion (Creeper and Buller, 2006). Co-habitating fish may serve as reservoirs of *S. iniae*, which is supported by the idea that fish can be subclinically infected until stressed (Bromage *et al.*, 1999).

*S. iniae* could also be spread through the faecal-oral route (McNulty *et al.*, 2003). *S. iniae* has been found in the intestine of barramundi up to 10 days post-infection, allowing release of the bacteria into the environment through excrement (Bromage and Owens, 2002). Infected faeces could lead to a large concentration of *S. iniae* in the farm environment, which can serve as a permanent reservoir for the bacteria (Perera *et al.*, 1997). *S. iniae* spreads more easily through mud than through water, so contaminated mud may be important in facilitating the spread of disease (Creeper and Buller, 2006). Mortality during the recent outbreak in Western Australia increased following a period of heavy precipitation, which caused silt and organic matter to contaminate the cages (Creeper and Buller, 2006). Heavy rainfall has also been

associated with *S. iniae* outbreaks in northern Australia, probably for the same reason: heavy rains cause infected mud and organic matter to become unsettled, introducing *S. iniae* directly into the fish environment.

It has been suggested that previous injury may increase the probability of infection by aiding in the establishment of *S. iniae* from the environment (Evans *et al.*, 2000). However, Bromage and Owens (2002) found injury to the epidermis to have no effect on the ability of the bacteria to enter the host. They propose that initial infection in farms is through the oral route, either by carrier fish, trash fish, cannibalism or the consumption of faeces. The oral route of entry appears to lead to a subacute infection, which allows ample *S. iniae* to be introduced into the environment for a longer period of time by the excrement of these fish. Enough infected excrement can lead to levels of bacteria in the environment capable of causing infection in fish through water-bourne exposure, which leads to acute infection and high mortality.

Transmission of the bacteria has occurred between wild and cultured fish, though the direction of transmission is not clear. Zlotkin *et al.* (1998) suggested that wild fish may transfer *S. iniae* to cultured fish after they found the same clone of the bacteria in both wild and cultured populations in and around farms along the Israeli Mediterranean shore. The wild population of spine foot (*Siganus rivulatas*) suffered mortalities due to *S. iniae* during the same periods as the cultured sea bream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*). The wild fish experienced a more severe systemic infection and experienced mortality earlier than the cultured fish, suggesting that the spine foot was more susceptible to *S. iniae* and could act as a natural indicator of disease. Strain relationships in this study suggested cross-infection, so the evidence is inconclusive as to whether the infection began in the wild or cultured stocks. *S. iniae* transmission between cultured and wild fish was again reported in 2002 in the Red Sea (Colorni *et al.*, 2002). Again, the cultured and wild fish were infected with the same strain of bacteria. As this was the first report of *S. iniae* in wild Red Sea fish, and because the imported cultured red drum (*Sciaenops ocellatus*) had previously been

diagnosed with *S. iniae* infections on several occasions, it is assumed that the bacterium was transmitted from the cultured fish to the wild populations (Colorni *et al.*, 2002).

Despite which direction *S. iniae* is initially transmitted, wild fish near sea cages are likely to serve as important reservoirs for the bacteria once they are infected. Organic matter, mud and seawater also appear to be reservoirs for bacteria (Creeper and Buller, 2006). However, considering that environmental levels of *S. iniae* at fish farms drop quickly following an outbreak (Owens, 2007), wild and co-habitating fish carrying the bacterium at subclinical levels seem to be the most likely reservoir of the bacteria that can lead to future outbreaks in times of stress.

### 2.8 Virulence Factors of *Streptococcus iniae*

Little is currently known about the pathogenesis of *Streptococcus iniae* (Shin *et al.*, 2006), but some factors have been found that may contribute to the virulence of this bacterium. *S. iniae* is capable of crossing the blood-brain barrier, which allows quick infiltration of organs in the host (Evans *et al.*, 2001). *S. iniae* α-enolase allows the bacteria to cross host tissue barriers by binding to the host plasminogen system, which normally defends the host by maintaining homeostasis and vascular potency (Kim *et al.*, 2007b). Localization of the bacteria in the olfactory organ and the brain olfactory lobe in fish, signalled by reluctance to feed 24-48 hours after infection, may be important in the pathogenesis of the disease (Evans *et al.*, 2001). The bacteria also grow rapidly in the intestine, where it is proposed that the bacteria produce extracellular and intracellular toxins (Seng and Colorni, 2002). There is some evidence that *S. iniae* may produce a cell surface Fc-binding factor for fish immunoglobulin, which would help the bacteria avoid the defence mechanisms produced by this region of Ig molecules (activation of complement and opsonisation) (Barnes *et al.*, 2003a).

The more virulent strains of *S. iniae* have an increased ability to avoid phagocytosis and oxidative killing in the blood (Fuller *et al.*, 2001; Neely *et al.*, 2002; Buchanan *et al.*, 2008). They also have the ability to invade host cells, with an affinity for uptake by macrophages and the ability to persist inside them, which may serve as a mechanism for

crossing the blood-brain barrier (Zlotkin *et al.*, 2003). The importance of intracellular survival was again shown by the research of Eyngor *et al.* (2007), as well as the ability of the bacteria to translocate through skin barriers in fish, which aids rapid entry into the host bloodstream and organs.

There is histological evidence that *S. iniae* produces potent exotoxins that help the disease progress. Activation of the immune system by bacterial exotoxins may lead to severe disseminated intravascular coagulation, indicated by fibrin deposits seen in the brain and spleen of infected fish (Bromage and Owens, 2002). Chemotactic activities of secreted and excreted extracellular products (ECP) of *S. iniae* were suggested to be involved in the pro-inflammatory responses of macrophages to localized *S. iniae* in organs (Perera *et al.*, 1997; Neely *et al.*, 2002). This hypothesis was confirmed by the work of Klesius *et al.* (2007), who found *S. iniae* ECP to be strong attractants of macrophages.

Virulent strains of *S. iniae* possess a polysaccharide capsule that helps the bacteria evade host immune responses (Barnes *et al.*, 2003b; Miller and Neely, 2005). Encapsulation allows the bacteria to resist phagocytosis and invasion by epithelial cells and macrophages, and this resistance is a key characteristic of disease-associated strains of *S. iniae* (Fuller *et al.*, 2001; Zlotkin *et al.*, 2003; Locke *et al.*, 2007a; Lowe *et al.*, 2007). A capsule operon (GenBank accession number AY904444) composed of several capsule synthesis genes has been identified for *S. iniae* (Locke *et al.*, 2007a; Lowe *et al.*, 2007). Strains with a deletion of the operon show reduced capsule formation and attenuated virulence *in vivo* even at 100 times the 100% lethal dose for the wild type strain, confirming that the capsule is essential to virulence (Locke *et al.*, 2007a).

Serotype II strains of *S. iniae* have evolved a capsule that provides more coverage of the cell surface and has different protein antigens than that of serotype I, allowing serotype II to evade the protective response elicited by vaccination with serotype I (Barnes *et al.*, 2003b; Lowe *et al.*, 2007). As they possess different capsular polysaccharides, serotype II strains have an increased ability to persist and multiply in phagocytes and

macrophages, causing elevated levels of apoptosis in these host cells (Lahav *et al.*, 2004). Clearly, *S. iniae* serotype II has evolved to proliferate and survive more successfully than serotype I in internal tissues.

While capsule expression is an important virulence factor for *S. iniae*, the optimum level of capsule expression is not completely understood. Lowe *et al.* (2007) discovered that reduced capsule expression was tolerated in some environments while overproduction of capsule can sometimes be detrimental. They concluded that capsule production in *S. iniae* is probably regulated for different host environments, which is important when considering the virulence of *S. iniae* strains grown in different animals or propagated in the laboratory on artificial media. A simple way to ascertain the relative virulence of *S. iniae* strains is to observe their buoyancy. Disease causing strains of *S. iniae* are more buoyant in broth than commensal strains, suggesting that the capsule or other aggregation inhibiting surface component is present in virulent strains (Bolotin *et al.*, 2007; Locke *et al.*, 2007a; Lowe *et al.*, 2007). Another interesting phenomenon that can be observed in the laboratory is that reduced capsule expression (and thus attenuated virulence) increases coccus chain length in *S. iniae* (Locke *et al.*, 2007a).

Screens for molecular virulence determinants have identified specific genes responsible for *S. iniae* virulence factors. Fuller *et al.* (2002) identified the gene locus for cytolysin production as the Streptolysin S (SLS) operon by comparing the activity of mutants for this gene to normal *S. iniae*. SLS expression in *S. iniae* is critical to disease pathogenesis as it works by producing direct cytolytic injury to fish cells and tissues by pore formation (Locke *et al.*, 2007b). SLS expression does not contribute to the establishment of the bacteria or resistance to phagocytic clearance (Fuller *et al.*, 2002; Locke *et al.*, 2007b), so it is not the only virulence factor required for the establishment of disease. Phosphoglucomutase (PGM) is another gene required for virulence (Buchanan *et al.*, 2005). The PGM enzyme is required for cell wall integrity, surface capsule expression and resistance to cationic antimicrobial peptides. It was found that *S. iniae* with mutated PGM genes could stimulate a protective immune response in fish, reducing later mortality by infection with normal *S. iniae* bacteria. A two-component

signal transduction system termed sivS/R was also found to be associated with capsule expression and resistance to clearance from blood, as well as the expression of SLS and other genes related to virulence (Bolotin *et al.*, 2007). The most recent virulence factor identified for *S. iniae* is an M-like fibrinogen-binding protein embedded in the cell surface of the bacteria. This protein, encoded by gene *simA*, provides resistance to phagocytic attack in fish and contributes to cellular adherence and invasion (Baiano *et al.*, 2008; Locke *et al.*, 2008).

There remains a need to identify more virulence factors for this bacterium to find targets for therapy and vaccine development. Some difficulties in this search are the diversity of strains and serotypes of *S. iniae*, and the fact that different environments may have a stronger effect on virulence than previously thought. Buchanan *et al.* (2008) recently found that SLS expression, resistance to antimicrobial peptides and the ability to invade host cells did not differ significantly in between virulent and avirulent strains of *S. iniae* in a hybrid striped bass model. These results suggest that identified virulence factors should be tested over a wide range of *S. iniae* isolates and in different species of susceptible fish.

## 2.9 Treatment Options

### 2.9.1 Vaccinations

Due to the devastating effects of *S. iniae* on fish stocks worldwide, the search for an effective vaccine against infection by this bacterium has been very important. A protective immune response was elicited in fish by a vaccine made from formalin-killed cells (FKC) of *S. iniae* with no adjuvant required (Eldar *et al.*, 1997a). Rainbow trout vaccinated with FKC of *S. iniae* were protected against experimental and natural infection with the bacteria for at least 4 months, with specific antibody production detected for 6 months. Mortality decreased from 53% to 4.5% with vaccination, even when only 75% of trout in the field trial received the vaccine. This shared protection suggests that the susceptibility of individuals is influenced by the immune status of the community, which can help reduce time and effort for vaccinations.

Use of the *S. iniae* FKC vaccine was successful for two years in Israel, doubling trout production (Eldar *et al.*, 1997a). However, regular use of this vaccine in Israel most likely led to the evolution of *S. iniae* serotype II, which is able to evade the protective response from vaccination against serotype I (Bachrach *et al.*, 2001). The selective pressure of the vaccine, combined with the fact that *S. iniae* was never completely removed from the fish stocks or environment (Eldar *et al.*, 1997a), caused the second serotype to emerge and take over (Bachrach *et al.*, 2001).

Evans *et al.* (2006) tested vaccination as a therapeutic measure in asymptomatic, subclinically infected hybrid striped bass. Injected vaccines containing concentrated *S. iniae* extracellular products with FKC increased survival in infected fish by rapidly clearing bacterial cells from the fish. This study showed that vaccination with *S. iniae* ECP may help control bacterial populations left from previous infections in fish.

The bacterial resistance and evolution caused by the overuse of vaccines illustrate the urgency of finding alternative solutions for treatment and prevention of *S. iniae* infection in fish, and also the importance of using these solutions only when necessary (and possibly in conjunction with other treatments) to slow further bacterial evolution.

### 2.9.2 Antibiotics

Fear of further bacterial evolution due to vaccinations led to more widespread use of antibiotics for prevention and chemotherapy against *S. iniae* in cultured fish. Erythromycin, spiramycin, doxycycline, enterofloxin and josamycin were found to be effective in prevention of *S. iniae* when in delivered orally to fish through medicated feed (Kasuda and Salati, 1999; Seng and Colorni, 2002). Treatment of *S. iniae* infection with fluoroquinolone compound and enrofloxacin may also reduce mortalities in fish (Abutbul *et al.*, 2004). Other antibiotics successful at treating infection with *S. iniae* are florfenicol (Yanong *et al.*, 2005), amoxicillin (Darwish and Hobbs, 2005), oxytetracycline (Darwish *et al.*, 2002), cefotaxime, ofloxacin, penicillin and vancomycin (Park *et al.*, 2009). Tricide™, a chelator-buffer that results in increased permeability and sensitivity to antibiotics, has been trialled in combination with neomycin and

oxytetracycline against *S. iniae*. Lower concentrations of antibiotics were required with this combination, and no resistant bacteria were recovered following serial passage trials (Wooley *et al.*, 2004).

Although current antibiotics may be effective in preventing epizootics caused by *S. iniae*, there are many problems concerning the long-term use of antibiotics in fish. Use of antibiotics over long periods often leads to drug resistance in bacteria, which in turn reduces the efficacy of the drugs. New antibiotics are not being developed quickly enough to replace those that are becoming less useful due to a slow in production driven largely by financial reasons (Spellberg *et al.*, 2004). Only three novel classes of antibiotics have been introduced to the medical field in the past four decades (Parisien *et al.*, 2008). Antibiotic residues on fish can also lead to transferred bacterial resistance and other risks for consumers (Abutbul *et al.*, 2004). Thus, prolonged therapy with antibiotics prevents the marketing of some fish late in the season because of antibiotic residues and long withholding periods (Ghittino *et al.*, 2003). Most importantly, no antibiotics are approved for wide-scale use in Australian commercial aquaculture (APVMA, 2010).

### 2.9.3 Green solutions

It has been suggested that chemical-free "green solutions" need to be developed for prevention of bacterial infections in fish (Abutbul *et al.*, 2004). The effects of dietary supplementation with inositol on resistance to *S. iniae* infection were tested in Nile tilapia (Peres *et al.*, 2004). However, inositol did not increase survival or resistance to the bacteria, nor did it enhance immunological responses by the fish. Another possible green solution, β-glucan, increases the innate immune response and resistance to bacterial infection in salmon (Robertsen *et al.*, 1990). However, dietary supplementation with β-glucan in tilapia had no effect on specific antibody response and resistance against *S. iniae* (Whittington *et al.*, 2005). Another hopeful green solution was levamisole, a synthetic antihelminthic drug that can act as a potent immunostimulant, enhancing bacterial resistance in some animals. However, levamisole

failed to reduce mortality in hybrid stiped bass from *S. iniae* infection when supplemented into their diet, and fish fed with high doses (1000 mg levamisole/kg diet) showed chronic toxicity signs (Li *et al.*, 2006).

Some successful green solutions may come in the form of prebiotics, nondigestible dietary ingredients that beneficially affect the host by selectively stimulating the growth and/or activating the metabolism of health-promoting bacteria in the intestinal tract (Gibson and Roberfroid, 1995). Li and Gatlin (2004) made experimental prebiotic food mixtures for hybrid striped bass: one containing only brewers yeast and another with a commercial mixture of autolyzed brewers yeast, dairy ingredient components, and dried fermentation products (Grobiotic™ AE). The prebiotics increased survival of fish challenged by *S. iniae* immersion and enhanced the immunological response of the fish (Li and Gatlin, 2004). Nucleotides and β-glucans may increase disease resistance in fish. In red-tail black sharks (*Epalzeorhynchos bicolor*), these immunostimulants decreased mortality from *S. iniae* infection in vaccinated and unvaccinated sharks, but not as significantly as vaccines alone (Russo *et al.*, 2006). Protective effects offered by prebiotics depend on many factors including genetics, nutrition, stress, water temperature and handling (Russo *et al.*, 2006).

Rosemary *Rosmarinus officinalis* has been tested as a green *S. iniae* treatment (Abutbul *et al.*, 2004). This plant is a rich source of active metabolites and shows natural antibiotic activity. *In vitro*, ethyl acetate extract from rosemary controlled the growth of *S. iniae* cells at first, but the bacteria made a full recovery after two days. *R. officinalis* was then mixed with food as crushed dried leaves or ethyl acetate extract and tested as a therapeutic in tilapia. Fish were fed *R. officinalis* for five days before inoculation with *S. iniae*, and then continued to receive the food mixtures. Both of the mixtures significantly reduced mortality over unsupplemented fish, but the treatment was not as effective as the antibiotic oxytetracycline.

### 2.9.4 Probiotics

The use of probiotics, bacteria that act beneficially to the host, is another treatment option that is attracting growing attention in aquaculture (Hagi and Hoshino, 2009). Live *Aeromonas sobria* cells isolated from a fish digestive tract were found to confer protection to rainbow trout against *S. iniae* mortality by stimulation of innate cellular immunity (Brunt and Austin, 2005). Currently, no other published studies have trialed beneficial bacteria against *S. iniae* infection, but probiotics have the potential to reduce mortalities due to the bacterium.

### 2.9.5 New treatment options

Bacteriophages and bacteriocins with specific antibacterial activity for *S. iniae* could increase the effectiveness of current control strategies in aquaculture, if not replace them completely. Research on bacteriophages has been ongoing for over a century, but it is only recently that these naturally occurring viruses been considered as therapeutics for aquatic pathogens. Recent advances in protein research have revealed the potential of bacteriocins in prevention and therapy of bacterial pathogens. These potential treatment options will be the topics of the remaining review.

## 2.10 Bacteriophages

Bacteriophages (phages) are simple viruses that infect bacteria. These infectious particles, like other viruses, are composed of nucleic acid and protein components (Campbell, 2003). Phages may have genomic RNA or DNA (single or double-stranded). The basic phage types are tailed, filamentous and icosahedral, all of which are classified into one order, *Caudovirales* (Ackermann, 2006b). Most described phages (96%) are dsDNA tailed phages classified into family by tail features: *Myoviridae* (contractile tail), *Siphoviridae* (long noncontractile tail), and *Podoviridae* (short tail) (Ackermann, 2006b; McAuliffe *et al.*, 2007; Skurnik *et al.*, 2007; Parisien *et al.*, 2008). All phages are

obligate intracellular parasites; they require bacterial cells to replicate (Jensen *et al.*, 1998). These viruses are one of the most abundant biological entities on the planet, with an estimated $10^{31}$ phages on earth (Ackermann, 2001).

### 2.10.1 How bacteriophages operate

The phage infection process is initiated when a phage interacts with cell surface receptor molecules on the host bacterium (Jensen *et al.*, 1998). Host receptors on bacteria that have been identified to date are composed of carbohydrates or proteins (Skurnik *et al.*, 2007). Following interaction with bacterial receptors, the phage inserts its nucleic acid into the bacteria. The next steps of the infection process differ depending on whether the phage is lytic or lysogenic.

### 2.10.2 Lytic bacteriophages

Lytic (virulent) phages take over the biosynthetic apparatus of the bacterial cell (ie. ribosomes and ATP generators) when the phage nucleic acid is inserted into the cell, triggering phage production. Around 100 progeny phage can be released when the bacterial cell envelope is disrupted by phage enzymes and the cell lyses. Most lytic phages employ the virolysin-holin system to hydrolyse the bacterial cell wall (Parisien *et al.*, 2008). The entire cycle, from recognition of host surface receptor molecules to the release of new phage particles from the lysed bacteria, can occur in as little as 40 minutes (Campbell, 2003).

### 2.10.3 Lysogenic bacteriophages

Lysogenic (temperate) phages do not follow the same infection process. Lysogenic phages integrate their genome into the host bacterial genome or the related membrane replicatory system, creating a prophage (Barksdale and Arden, 1974). Lysogenic phages change the bacterial genome architechture, and can affect bacterial fitness by several processes, including transduction, gene disruption, genome rearrangements, and the

introduction of fitness factors (Brussow *et al.*, 2004). Many lysogenic phages are known to confer virulence factors to host bacteria via lysogenic conversion (Carlton *et al.*, 2005). Lysogenic conversion may also be brought about by changes in the synthesis of bacterial products caused by prophage genes (Barksdale and Arden, 1974). Most importantly, lysogenic phages provide host bacterial immunity to infection by related lytic phages, a process termed superinfection immunity (Barksdale and Arden, 1974; Jiang and Paul, 1998; Day, 2004).

Globally, an estimated $2 \times 10^{16}$ genetic modification events occur in bacteria due to phage activity every second (Bushman, 2002). It is becoming apparent that phage-mediated genetic exchange does not just occur within closely related bacteria; prophages appear to have a much greater effect on the ecology and evolution of bacterial pathogens than originally thought (Chen and Novick, 2009).

It is important to note that not all temperate phages are lysogenic; some phages enter a state of pseudolysogeny (or stable carrier state) in which phage genes can be carried by the bacterial cell without being integrated into the bacterial genome (Barksdale and Arden, 1974). Often these pseudolysogenic phages are liberated slowly from the host cell without causing cell lysis. Pseudolysogenic phages can be completely removed from bacterial cells by cultivating the cells in antiphage serum, while truly lysogenic phages are resistant to removal by this method (Barksdale and Arden, 1974).

Lysogenic phages are often highly stable in the prophage form, and have been observed to remain with the bacterial genome for over 70 years with repeated subculture (Barksdale and Arden, 1974). Lysogenic phages can eventually become lytic, even after several generations of bacterial replication, and cause the cell to lyse (Douglas, 1975). Induction of most prophages can be achieved by a range of environmental stressors including irradiation, dessication and nutrient starvation (Day, 2004). Some phages are easily induced, while some show only a slight increase following induction or cannot be induced by certain methods (Barksdale and Arden, 1974). Inducibility can depend on both the phage and the host bacterial strain (Loessner *et al.*, 1991). Lytic

phages do not possess the genetic factors required for genomic integration, and thus do not produce prophages in bacteria (Carlton *et al.*, 2005). For this reason, lytic phages are safer for use in therapy against bacterial infections (Carlton *et al.*, 2005).

The rate of phage replication of both phage types once in the lytic cycle is dependent on the adsorption rate, latent period, burst size, temperature, ratio of phage to bacteria in the environment, density and physiological state of bacterial population (Levin *et al.*, 1977). High bacterial cell density can affect phage replication in two ways: 1) the bacteria can exhaust the environmental nutrients, which will decrease bacterial growth rates, and thus decrease phage replication, or 2) phage replication will be increased because the time required for a phage to encounter a host cell is lowered (Bull *et al.*, 2002).

The surface structures on bacteria that serve as receptor molecules for a phage determine the host range of each phage (Skurnik *et al.*, 2007). Most studies report that phages are highly specific for host cell receptors of a certain structure, and subsequently do not infect antigenically unrelated bacterial cells (Barrow and Soothill, 1997; Jensen *et al.*, 1998; Summers, 2001; Weber-Dabrowska *et al.*, 2001). However, the specificity of many studied phages may be biased by enrichment methods with specific hosts (Jensen *et al.*, 1998). This specificity by enrichment is an advantage when customized phages are required to fight a particular strain of bacteria. Multiple host enrichment is effective for the isolation of broad-host-range phages (Jensen *et al.*, 1998).

### 2.10.4 Bacteriophage research

Bacteriophages were first discovered in 1915 by English bacteriologist Frederick Twort (Twort, 1915). Phages were also supposedly found and described independently by French-Canadian microbiologist Felix d'Herelle in 1917 (Duckworth, 1976). There is an interesting (if not entertaining) controversy surrounding the question of who deserves to be recognized as the first to discover phages (Duckworth, 1976). Regardless, d'Herelle was the first to clearly describe the ability of phages to cause epizootics in

bacteria and to suggest that phages are viral in nature and would make an excellent tool to promote recovery from infections (Summers, 2001).

Many early trials of phage therapy (in the lab and in the field) during the 1920's and 1930's appear successful, reporting effective treatment of bacterial dysentery, surgical wound infections, and diarrheal disease, but these trials lack the proper controls of today's scientific standards (Summers, 2001; McAuliffe *et al.*, 2007). Development of phage therapy was practically abandoned with the rising use of antibiotics during World War II, except for some ongoing research in the Soviet Union and Eastern Europe (Merril *et al.*, 1996; Summers, 2001; Weber-Dabrowska *et al.*, 2001). Antibiotics were easier to produce, had a broader spectrum, and were more stable than phages. Phage research was also tainted politically in the "Western world" due to the continued research efforts in the Soviet Union. It may have also been thought that the obstacle of phage-resistance in bacteria was larger than it appears today (Summers, 2001).

Continued research on phages following WWII, though reduced, provided some very useful results. In 1940, phages were visualized using electron microscopy, confirming that they are in fact a virus-like particle rather than some sort of enzyme (Summers, 2001). The necessity of matching phages to their specific target for therapy was realized in 1987 (Smith *et al.*, 1987). Phage research also helped drive the development of modern molecular biology, ie. the first completely sequenced genomes were from bacteriophage φX174 (Sanger *et al.*, 1978). The emergence of multiresistant bacteria and low rate of discovery of novel antibiotics over the last 20 years has illuminated the need for alternative treatments for bacterial infections, and thus interest in phages has recently become reactivated (Summers, 2001; Weber-Dabrowska *et al.*, 2001).

Today there are several different antibacterial strategies derived from phages including lethal agent delivery systems (engineered phage deliver lethal substances to the bacterial cells), enzybiotics (host-specific phage-encoded lytic enzymes introduced to combat bacteria without the whole phage), bacterial ghosts (vaccination technique in which the cells are attacked by phage lysins, causing the loss of cell contents while the cell wall

remains intact), and whole phage therapy (introducing viable phage to attack the infecting bacteria) (Hermoso *et al.*, 2007; Petty *et al.*, 2007).

### 2.10.5 Bacteriophage therapy (lytic whole phages)

One promising solution for the problems caused by *S. iniae* in aquaculture is phage therapy. There are many advantages to whole phage therapy, making it one of the leading alternatives to antibiotic treatment for the control of bacterial infections (Summers, 2001; Bull *et al.*, 2002; Campbell, 2003). Phages are naturally abundant, have a specific host range and replicate quickly in the presence of host bacteria. Development and production of phages is faster and cheaper than antibiotics after the recognition molecules are known. Phage therapy also appears to cause lower levels of bacterial resistance than current control measures used for *S. iniae*.

The natural abundance of phages is an important advantage to phage therapy because it helps ensure that treatment is safe for use in animals and humans, and it means phage therapy will not leave residues or require with-holding periods like many antibiotics. Phages are quite common in all habitats where bacteria live and grow. In aquatic ecosystems, phage-like particles are more abundant than prokaryotic cells (Campbell, 2003; McAuliffe *et al.*, 2007). In fact, phages may be the most widely distributed biological entity on the planet, estimated to be present in concentrations of 10 million per cubic centimeter in any environment where bacteria or archaea proliferate (McAuliffe *et al.*, 2007). Phages are normally present in humans and animals (Breitbart *et al.*, 2003), and are routinely consumed in significant numbers in our food (Skurnik *et al.*, 2007). Unlike many other therapies, the introduction and spread of a phage in a given environment is a natural process (Carlton *et al.*, 2005). As phages are so abundant in nature, it should be possible to isolate a substantial amount of phages able to lyse different strains of bacteria (Bull *et al.*, 2002).

Side-effects with phage therapy should be uncommon because phages and their products do not affect eukaryotic cells (Matsuzaki *et al.*, 2005). Tests performed on fish serum

following phage therapy trials detected no neutralizing antibodies against the phage used (Nakai *et al.*, 1999; Park and Nakai, 2003), supporting the safety of using phage therapy in fish aquaculture.

As discussed previously, the specificity of phages (whether natural or achieved by enrichment methods) can be an advantage for phage therapy. Phages specific to a bacterial capsule are more effective at preventing mortality in animals infected with that bacteria than non-specific phages (Bull *et al.*, 2002). The narrow host range of phages also means that they attack only the desired bacterial cells and leave commensal flora and eukaryotic cells unharmed (Barrow and Soothill, 1997; Park *et al.*, 2000; Weber-Dabrowska *et al.*, 2001; Skurnik *et al.*, 2007). It is important that phages with a broad host range can be made more specific by enrichment because this implies that phages can be used for a variety of issues that may arise with bacterial infections. For example, if the infecting bacterium is unknown, it is possible that delivery of a specific phage cocktail could result in the treatment, and possibly the identification, of the bacteria causing infection (Levin and Bull, 2004).

Phages multiply their population as they lyse bacterial cells, meaning that a single dose of phage may be all that is required for protection to be conferred, in contrast to the many doses of antibiotics required for treatment (Alisky *et al.*, 1998; Skurnik *et al.*, 2007). A small dose of phage can treat a larger, lethal dose of bacteria due to *in vivo* replication of phages (Barrow and Soothill, 1997). Phage replication *in vivo* is important because it implicates the phage as the causative agent of bacterial lysis (Bull *et al.*, 2002; Nakai and Park, 2002). Phages will not replicate unless they are lytic against the particular bacteria present, so there is no risk of side effects if the wrong phage is used for treatment (Barrow and Soothill, 1997).

Phage therapy may not have as many problems concerning bacterial resistance as other antimicrobial treatments. As phages are biological entities, they have the ability to mutate and overcome bacterial resistance. Co-evolution between phages and bacteria has been studied *in vitro* (Alisky *et al.*, 1998; Levin and Bull, 2004). Phage-resistant

bacteria have often been found to have reduced virulence. Phage-resistant variants of *Pseudomonas plecoglossicida* induced *in vitro* lack virulence in ayu *Plecoglossus altivelis* (Park *et al.*, 2000). These bacteria showed little development of phage-resistance *in vivo*; bacteria isolated from phage treated fish were still susceptible to phages used in the treatment (Park *et al.*, 2000). Phage-resistant *Escherichia coli* that have emerged in phage therapy of mice and calves have been less virulent (Smith and Huggins, 1982; Smith and Huggins, 1983). One explanation for the attenuated virulence of phage-resistant bacteria observed in these studies is that the host cell receptor used by the phages to attach to the bacteria was the same surface component associated with bacterial virulence (Smith *et al.*, 1987; Park *et al.*, 2000). However, some phage-resistant bacteria isolated following *in vivo* phage therapy in fish have retained high virulence, a finding that supports the importance of using several different lytic phage for treatment in the form of a phage cocktail (Iwamoto *et al.*, 2008).

Economically, phage therapy may have an advantage over current antibacterial treatments; the cost of developing phage treatment is cheaper than developing new antibiotics (Skurnik *et al.*, 2007; Parisien *et al.*, 2008). As phages are naturally abundant and replicate in the presence of host bacteria, it should not only be cheaper to develop most phage products, it should be faster. These economic and timing factors are yet to be proven, but it seems likely that phage therapy could become an easier treatment option for aquaculturalists worldwide.

#### 2.10.5.1 Improvements to phage therapy

As research of phage therapy continues, more is learned about the methods needed to make this type of treatment more effective. Phage cocktails, precise timing of therapy and delivery of phages are all important to the success of phage therapy. The use of a two phage cocktail containing one lytic phage specific for the primary bacteria and one lytic phage specific for the bacteria resistant to the first phage may be effective for treatment as this cocktail would prevent the resistant strain from taking over during treatment (Smith *et al.*, 1987). A mixture of two or more phages has previously proven

more successful at inhibiting bacterial infection than each individual phage in treatment of *P. plecoflossicida* in ayu (Park and Nakai, 2003).

The optimal timing of phage therapy is still being determined, and it is possible that it may be different for each animal and for each bacterial strain. Mortality in mice infected with *E. coli* can be prevented when phage φLH is administered immediately following bacterial infection (Bull *et al.*, 2002). However, *E. coli* become refractory to phage φLH when it is administered just eight hours after infection, even when no disease signs are present in the mice. Problems with late treatment are probably due to a reduced susceptibility of the bacteria (Bull *et al.*, 2002). Slow growing bacteria are less likely to allow for adsorption and replication of a phage (Adams, 1959; Stent, 1963), so if phage treatment is delayed for too long, the bacterial population may become dense enough to slow its growth and reduce phage efficacy, as discussed previously (Bull *et al.*, 2002). Phage treatment for *P. plecoglossicida* in cultured ayu was effective even 24 hours after infection if the fish experienced systemic infection, though the lowest mortality was measured in fish receiving phages one hour post-infection (Park *et al.*, 2000).

Previous findings suggest that the delivery of phages to infected organisms may be fairly simple, but require some understanding of the bacteria being treated. In aquaculture, phages are able to invade fish from the surrounding environment since they can be found in the kidneys of fish immersed in a phage bath (Nakai and Park, 2002). Phages are also capable of moving to all parts of the body were bacteria are located once they have entered the animal (Barrow and Soothill, 1997), though it is important to note that some bacteria may localize in areas that may not provide an optimal environment for phage replication (Skurnik and Strauch, 2006). Phage treatment is most successful when delivered through the same route as bacterial inoculation (Nakai and Park, 2002).

Phage therapy may be more efficient if long-circulating phage mutants are used. Even when no antibody response is present, the mammalian reticuloendothelial system is able to rapidly eliminate phages from the circulatory system (Merril *et al.*, 1996). Using a

serial-passage technique to select for phages able to survive longer in the circulatory system should result in phages that are not only more abundant in the animal to fight infection, but also have more capability as antibacterial agents (Merril *et al.*, 1996). However, long-circulating phages may not be necessary for phage therapy in aquaculture, as the capacity of the fish immune system to clear phages from the circulatory system is not yet well understood.

#### 2.10.5.2 Previous successes with phage therapy

Phage therapy is repeatable and can be successful in treating bacterial infections *in vivo* (Bull *et al.*, 2002). Phages have been successful in the treatment of mice infected with *E. coli* and *E. faecium* (Smith and Huggins, 1982; Biswas *et al.*, 2002) and also *Staphylococcus aureus* (Matsuzaki *et al.*, 2003). Phages have also been tested successfully against animal and zoonotic pathogens in cattle, sheep, chickens and pigs. An excellent comprehensive review of phage therapy in these animals has recently been published (Johnson *et al.*, 2008). Phages have been used in humans for treatment of long-term bacterial infections of drug-resistant bacteria, and have produced an up-regulation of the human immune response to these infections (Weber-Dabrowska *et al.*, 2001). For example, infections with *Streptococcus* spp. were treated successfully in humans using phage therapy during 1974 Soviet experiments, though like many early reports on phage therapy, the results may not be completely reliable or reproducible due to a lack of scientific rigour (Alisky *et al.*, 1998). However, in 1991, clinical improvement was again found in human patients infected with *Streptococcus* spp. when treated with phages (Alisky *et al.*, 1998).

In February 2006, the United States Environmental Protection Agency approved the use of phages on tomato and pepper crops (Anon, 2006b). In August of the same year, the United States Food and Drug Administration (FDA) approved the use of a cocktail of six phages on meat products for the treatment of *Listeria monocytogenes*, which causes the contamination of meat and poultry products (Anon, 2006a). This marked the first

occasion that the FDA had approved a phage as a food additive. This approval suggests that use of phages in commercially grown fish should also be safe for consumers.

#### 2.10.5.3 Phage therapy in aquaculture

The earliest known study of bacteriophage activity against a fish pathogen was a characterization of phages specific for *Aeromonas salmonicida* (Paterson *et al.*, 1969). Some early trials of phage therapy in aquaculture focused on the control of pathogens like *Edwardsiella tarda* in the aquatic environment (Wu and Chao, 1982). Phages have since been isolated against other aquatic pathogens affecting molluscs, crustaceans and fish including *Vibrio vulnificus* (DePaola *et al.*, 1998), *Vibrio harveyi* (Vinod *et al.*, 2006; Karunasagar *et al.*, 2007; Crothers-Stomps *et al.*, 2009), *Flavobacterium psychrophilum* (Stenholm *et al.*, 2008), *Edwardsiella ictaluri* (Walakira *et al.*, 2008), *Pseudomonas plecoglossicida* (Park *et al.*, 2000) and *Lactococcus garvieae* (Park *et al.*, 1997).

Recent studies concerning the control of bacterial diseases in fish aquaculture have shown phage treatments to reduce mortality in infected animals and provide increased resistance to future bacterial infection (Nakai *et al.*, 1999; Park *et al.*, 2000; Nakai and Park, 2002; Park and Nakai, 2003). Phage therapy was tested in cultured yellowtail (*Seriola quinqueradiata*) infected with *Lactococcus garvieae*, and resulted in higher survival rates of the fish when phage were injected soon after the bacteria (Nakai *et al.*, 1999). Oral administration of phages has proved to be successful in other tests on fish (Nakai and Park, 2002). Phage therapy was trialed in a commercial fish culture pond of ayu (*Plecoglossus altivelis*) that was severely infected with *Pseudomonas plecoglossida* (Park and Nakai, 2003). Unfortunately, this field trial lacked a control pond, but the results are still encouraging for the use of phage therapy in aquaculture at a commercial scale. Mortality of fish decreased daily, paralleling the increase in phage concentration within the fish. The continued presence of phages in these fish signals *in vivo* phage-induced bacterial lysis (Park and Nakai, 2003), considering that phages were found to disappear from the organs of healthy, uninfected ayu within 12 hours (Park *et al.*, 2000).

The use of phages to control fish diseases appears promising not only because of some apparently successful studies performed in aquaculture previously (Nakai and Park, 2002), but also because the aqueous environment provides good conditions for phage therapy. Physiological contact in aqueous media is natural for pathogens and phages that have evolved in this environment, and can be easily tested in laboratory culture conditions (Summers, 2001). Thus, a reduction in the environmental load of the pathogen being treated is an added advantage of phage therapy in aquaculture (Morrison and Rainnie, 2004).

It is important to mention that failures of phage therapy to control pathogenic bacteria in fish have also been published. Verner-Jeffreys *et al.* (2007) found that while phages specific for *Aeromonas salmonicida* produced no adverse effects in Atlantic salmon (*Salmo salar*) or rainbow trout (*Oncorhynchus mykiss*), phage therapy did not affect mortality rates in these fish following infection with *A. salmonicida*, despite viability of the phages against the bacteria *in vitro*. These failures may have occurred for any number of reasons, including the timing or route of phage administration, the dose rate of the phages, or a slow rate of bacterial or phage replication *in vivo*.

#### 2.10.5.4 Phage therapy for *Streptococcus iniae*

Current research suggests that phage therapy could be an efficient alternative treatment for *S. iniae* infections. Phages infecting *Streptococcus* species, frequently from the family *Siphoviridae*, have been identified and characterised (Stanley *et al.*, 1997; Ackermann, 2006a). Also, phage therapy has been tested successfully in cultured fish infected with *Lactococcus garvieae*, which is closely related to *S. iniae* (Nakai *et al.*, 1999).

Phage therapy has been trialled in Japanese flounder using lytic phage against Japanese isolates of *S. iniae* (Matsuoka *et al.*, 2007). However, because reports suggest that Asian isolates of *S. iniae* are phenotypically different from isolates in North America (Lau *et al.*, 2006), and North American isolates were found to be similar to Australian fish

isolates (Nawawi *et al.*, 2008), it is highly possible that phages lytic against Japanese isolates would not be viable in phage therapy against Australian isolates of the bacteria. The only *S. iniae* isolates trialled with the Japanese lytic phages that were not susceptible to the phage activity were isolated from Korea (Iwamoto *et al.*, 2008). Future research will hopefully identify and characterise bacteriophages capable of lysing the majority of global *S. iniae* strains and find a way to use this phage in therapy for cultured fish.

### 2.10.6 Whole phage prophylaxis

Phages are capable of inhibiting bacterial growth in water, and therefore have been suggested as a potential prophylactic for bacterial infection and transmission in aquaculture (Park *et al.*, 2000; Morrison and Rainnie, 2004). Environmental prophylaxis should only require one application of phages as long as they continue to encounter host bacteria (Levin and Bull, 2004). However, phages should probably not be used as a prevention tool due to the potential development of resistant bacteria and lysogeny. Low host cell density favors lysogeny (Paul *et al.*, 2002), so use of phages when host bacteria are present at very low densities in the environment or animal could lead to lysogeny, and perhaps resistance to future phage therapy.

### 2.10.7 Lysogenic phages in therapy

Lysogenic phages can be genetically modified for use in research and therapy of bacterial pathogens. The possibility exists for genetic modification to a lysogenic phage to render it permanently lytic by the removal of repressor and integration genes. The prophage repressor gene, which prevents the initiation of lytic gene expression (ICTV, 2005), could potentially be removed from the lysogenic phage. The expression of many genes related to viral function are silenced by repressor genes of the lysogenic genome, thus determining the stability of lysogeny (Barksdale and Arden, 1974).

Lysogenic phages can be used as vectors for DNA or protein-based vaccines, or as agents of gene transfer for therapeutic purposes (Serwer *et al.*, 2007). Genetically engineered lysogenic phages can deliver DNA encoding bactericidal proteins (suicide systems) to bacteria through phagemids (Westwater *et al.*, 2003). If addiction modules can be identified in the genome of *S. iniae*, toxins from these modules could be delivered to the bacteria by lysogenic phages, resulting in cell death (Westwater *et al.*, 2003). The phage delivery systems trialled previously have been found to target host bacteria at a high frequency (Westwater *et al.*, 2003). A lysogenic phage with a broad activity spectrum within *S. iniae*, would be excellent for phage delivery or other genetic modifications because it may not be necessary to identify the specific strain of bacteria prior to treatment.

Engineered non-lytic phages can also act as strong adjuvants for bactericidal antibiotics, and enhance the activity of antibiotics by expressing proteins to target specific gene networks (Lu and Collins, 2009). These phages can enhance killing of antibiotic-resistant pathogens and reduce the rate of appearance of newly antibiotic-resistant mutants when used in combined therapy.

### 2.10.8 Other phage applications

Phages can play a role in the diagnosis of bacterial pathogens through phage typing, in which specific phages are added to a lawn of an unknown pathogen to determine the species or even strain (Clark and March, 2006). Phage typing could be particularly helpful with *S. iniae* due to the difficulty in identifying the bacteria.

Lytic or lysogenic phages can be used as vaccine delivery vehicles, either by displaying vaccine antigens directly on their surface (phage-display vaccination), or by carrying a DNA expression cassette in their genome (Clark and March, 2006). Phage structural proteins have been used to create virus-like particles (VLP) to deliver vaccines, such as the FDA approved human Papillomavirus vaccine (Bundy *et al.*, 2008). VLP are phage capsid heads which lack the phage genome, and their size and stability make them

perfect for recognition by the immune system (Reddy *et al.*, 2006). Phage tail-like bacteriocins are another product composed of phage fragments, which are produced naturally by a number of Gram-negative bacteria (Daw and Falkiner, 1996). These high-molecular weight particles form pores in the bacterial cell wall which eventually lead to loss of ions and cell death (Strauch *et al.*, 2001).

Enzybiotics are phage endolysins, or enzymes that lyse peptidoglycan in bacterial cell walls in order to release phage progeny after multiplication inside the cell. Enzybiotics are usually specific for the host bacteria of the phage, and can be purified for use without whole phage. In the case of Gram-positive bacteria, enzybiotics do not need to be inserted into the cells due to the accessibility of the peptidoglycan layer (Hermoso *et al.*, 2007). These lysins irreversibly damage bacterial cell walls on contact (Hermoso *et al.*, 2007), and therefore could be an excellent therapeutic tool for *S. iniae* outbreaks. The peptidoglycan layer is an essential bacterial structure, and thus no resistance to phage endolysins has been observed (Hermoso *et al.*, 2007).

It is important to note that there is still much to be learned about the process of phage therapy, especially *in vivo*. Phages will probably not replace antibiotics in the fight against harmful bacteria, at least not in the near future (Mattey and Spencer, 2008). However, whole phage therapy or other phage-derived treatments used in conjunction with antibiotic or vaccination routines may provide more complete treatment and prevention of bacterial pathogens (Hermoso *et al.*, 2007; Petty *et al.*, 2007). The combined use of phages with antibiotics should be more effective than either treatment alone because fewer resistant bacteria should be able to emerge due to the need to evolve different resistant mechanisms for both treatments (Petty *et al.*, 2007; Lu and Collins, 2009).

### 2.11 Bacteriocins

Bacteriocins are a microbial defense system; these antimicrobial peptides are produced and secreted by bacteria to help them compete (Riley and Wertz, 2002). Bacteriocins

are widely defined as ribosomally synthesized antimicrobial peptides with a bactericidal mode of action (Tagg *et al.*, 1976). Bacteriocins inhibit the growth of closely related bacteria, conferring a selective advantage to the producing organism by reducing competition for its specific niche (De Vuyst and Vandamme, 1994). Bacteriocin production is normally regulated by cell-density and quorum-sensing, enabling the producing bacteria to turn on bacteriocin production when competition for nutrients becomes severe (Eijsink *et al.*, 2002).

The relatively narrow killing spectrum of bacteriocins is what differentiates these substances from traditional antibiotics (Riley and Wertz, 2002). Bacteriocins also differ from bacteriophages because bacteriocins can diffuse through solid media and do not replicate within their target bacteria (Tagg *et al.*, 1976). Bacteriocins are produced by both Gram-negative and Gram-positive bacteria, but these substances are dissimilar in several ways. The most important contrast is that the production of bacteriocins by Gram-positive bacteria does not kill the producing cell, whereas bacteriocin production is lethal for Gram-negative bacteria (Riley and Wertz, 2002).

### 2.11.1 Bacteriocin research

The first reported discovery of a bacteriocin was in 1925, when Gratia (1925) noted that a strain of the Gram-negative bacteria *E. coli* produced a highly specific antibiotic against other strains of the species. This was the first description of a colicin, which are the most extensively studied bacteriocins (Riley and Wertz, 2002). Upon later discovery that similar agents were produced by other bacteria, the name 'bacteriocine' was proposed for all highly specific bacteria-produced proteins with antimicrobial activity against closely related strains (Jacob *et al.*, 1952). While the bacteriocins of Gram-negative bacteria were the first to be described and studied, those of Gram-positive bacteria are also diverse and are the focus of much bacteriocin research.

The majority of Gram-positive produced bacteriocins identified to date are produced by lactic acid bacteria, and most of these bacteria belong to the genus *Lactococcus*

(Guinane *et al.*, 2005). Bacteriocins produced by lactic acid bacteria are currently classified mainly into two classes: lantibiotics which have undergone post-translational modifications (class I), and non-modified heat-stable bacteriocins (class II). Nisin, the most intensively studied lantibiotic, is produced in six natural forms by *Lactococcus lactis* species from fermented food origins and is also produced by *Streptococcus uburis* (Wirawan *et al.*, 2006; Field *et al.*, 2008). Nisin was first described in the 1920s (Rogers, 1928) and displays a relatively wide activity spectrum (Moreno *et al.*, 2000). It is currently the only lantibiotic approved for commercial use (Guinane *et al.*, 2005), and is approved as a food preservative in 50 countries (Delves-Broughton, 2005).

Current bacteriocin research is largely driven by the growing use of bacteriocins in food preservation and also by the search for novel antibacterial solutions for antibiotic resistant human pathogens. These interests have led to a dramatic growth in the bacteriocin field of research and thus the discovery and characterisation of numerous novel bacteriocins (Eijsink *et al.*, 2002; Cotter *et al.*, 2005; Nes *et al.*, 2007). It is thought that nearly all bacteria produce at least one bacteriocin, but they simply have not been discovered and documented because they have not yet been searched for (Klaenhammer, 1988).

### 2.11.2 Characterisation of bacteriocin-like inhibitory substances

Bacteriocins are generally categorized by producing strain, common resistance mechanisms, peptide structure, and mode of action (Parisien *et al.*, 2008). Characterisation of a bacteriocin-like inhibitory substance (BLIS) is necessary for proper identification, categorization and to confirm whether the substance is a true bacteriocin.

An important step in the characterisation of a BLIS is to determine heat, pH and enzyme stability of the antimicrobial activity. Enzymes used to test BLIS sensitivity are normally proteolytic, including proteinase K, α-chemotrypsin, trypsin, and pepsin, but glycolytic and lipolytic enzymes are also sometimes included in tests to be sure the BLIS is protein in nature.

The mode of action or activity of a bacteriocin can be generally established as either bacteriostatic or bactericidal by the use of optical density measurements or viable cell counts of the indicator cultures over time. A reduction in these two factors indicates a bactericidal mode of action, which can be further specified as bacteriolytic by the use of transmission electron microscope (TEM) studies or measurement of intracellular enzyme release from sensitive cells (Morgan *et al.*, 1995; Martinez-Cuesta *et al.*, 2000). A loss of cell viability can occur more rapidly than a reduction in optical density of the culture, and this phenomenon suggests that lysis is caused indirectly by the bacteriocin (Martinez-Cuesta *et al.*, 2000). Many bacteriocins kill bacteria by inhibiting cell wall biosynthesis, by degrading the nucleic acid of the bacteria by ribonuclease or deoxyribonuclease activity, or by binding to the bacterial receptor, provoking dispolarization and perforation of the cytoplasmic membrane, inducing membrane perturbations (Ackermann, 2006b; Parisien *et al.*, 2008).

The antagonistic activity of a bacteriocin can be measured by any number of assay methods, performed in both liquid and solid media. Solid media activity assays include disc diffusion (Bhunia *et al.*, 1988), spot-on-lawn (Hoover and Harlander, 1993), and well-diffusion assays (Barefoot and Klaenhammer, 1983), and the most common liquid media activity assays are variations of the microtitre plate assay (Geis *et al.*, 1983). It is important to identify a consistent indicator strain to use in activity assays for characterisation, but most of these assays can also be used to test the activity spectrum of the bacteriocin with the use of many related or unrelated bacterial strains.

Numerous different techniques have been used to purify bacteriocins, including phase extractions, mass spectrometry, absorption-desorption, liquid chromatography and sodium dodecyl suphate polyacrilamide gel electrophoresis (SDS-PAGE). Pingitore and colleagues (2007) reviewed these techniques and others, most of which are normally preceded by an ammonium sulphate precipitation step to concentrate the bacteriocin. Filters with high protein affinity have also been used to collect and purify bacteriocin proteins (Morgan *et al.*, 1995).

Bacteriocins are often associated with plasmids or transposons (Broda, 1979; Riley and Wertz, 2002). Plasmid association can be confirmed by plasmid curing, or removal of the plasmid from daughter cells, normally by use of chemical antimicrobials (Shehane and Sizemore, 2002; Melancon and Grenier, 2003).

If the characterised BLIS appears identical to a previously identified bacteriocin, in many cases a simple PCR can confirm the identity. There are several published primer sets to detect structural genes of many identified bacteriocins produced by lactic acid bacteria (Rodríguez *et al.*, 2000). A web-based bacteriocin genome mining tool, BAGEL, has also been developed to identify bacteriocins through knowledge-based databases (de Jong *et al.*, 2006).

### 2.11.3 Bacteriocins in treatment of bacterial infections

Bacteriocins are recognized as potentially useful agents in the control of bacterial infections due to their effectiveness, non-toxicity and relatively cheap production (Brook, 1999; Reid *et al.*, 2001; Riley and Wertz, 2002). In the aquaculture industry, bacteriocins or their producing cells (in the form of probiotics) are anticipated as being effective replacements for antibiotics and chemotherapeutics to fight infectious diseases (Hagi and Hoshino, 2009).

### 2.11.4 Possible treatment issues

One concern about using bacteriocins to treat pathogenic bacteria is that cross-resistance with common antibiotics may occur. However, bacteriocin structure and mode of action appears to differ enough from most antibiotics to avoid this problem; within the lantibiotics, no cross-resistance has been found with any of the major classes of antibiotics used medically (Dawson, 2007). Spontaneous resistance of bacterial cells to lantibiotics has been observed, though a high multiplicity of infection was necessary to induce a significant frequency of resistance (Ming and Daeschel, 1993). The cell membrane mutations necessary for developed resistance to pore forming lantibiotics

could reduce pathogenicity in the bacteria, similar to bacteria with developed phage resistance (Smith *et al.*, 1987; Park *et al.*, 2000).

### 2.11.5 Bacteriocins and *Streptococcus iniae*

Kim and Austin (2008) reported a heat-stable bacteriocin from *Carnobacterium divergens* which produced inhibitory activity against both Gram-positive and Gram-negative bacteria, including *S. iniae*. To date, there are no published studies on other bacteriocins with action against *S. iniae*. A few other bacteriocins have been isolated from fish and fish products (Stoffels *et al.*, 1992; Pilet *et al.*, 1995; Duffes *et al.*, 1999; Yamazaki *et al.*, 2003; Campos *et al.*, 2006; de Kwaadsteniet *et al.*, 2008), however these studies tested bacteriocin activity against *S. aureus*, *L. monocytogenes*, and other pathogens important in food spoilage and human infection, and have not included fish pathogens such as *S. iniae* in their sensitivity tests.

There are also no documented characterisations of bacteriocins produced by *S. iniae*, though a large number of antimicrobial peptides have been isolated from other streptococci (Nes *et al.*, 2007). However, SLS, one of the virulence factors for *S. iniae*, is designated as a bacteriocin-like toxin (Fuller *et al.*, 2002). Hagi and Hoshino (2009) isolated at least two *S. iniae* strains from fish with antagonistic activity against different species of *Aeromonas*, but it was concluded that the activity was probably caused by the production of organic acids or metabolites, rather than bacteriocin-like inhibitory substances.

## 2.12 Conclusion

Since its discovery in 1976, *S. iniae* has spread to become one of the most important Gram-positive bacterial pathogens in global aquaculture. With the development of new serotypes of this bacterium due to vaccinations and the potential spread of antibiotic resistance, it is essential to uncover new antimicrobial agents to reduce *S. iniae* epizootics in fish. Bacteriophages, in the form of whole lytic phage therapy or purified

phage products, show great promise as new therapeutic agents in aquaculture. Bacteriocins and/or their producing bacterial strains may also serve as excellent alternatives for the treatment and prevention of *S. iniae* in fish, as purified proteins or probiotics. The discovery of lytic phages and bacteriocins specific for a broad range of *S. iniae* isolates will be the first step in studying the efficacy of these agents against the pathogen *in vivo*.

# CHAPTER THREE

# GENERAL MATERIALS AND METHODS

## 3.1 Bacteriology

### 3.1.1 Trial bacterial isolates

The bacterial isolates used during this study include strains of *Streptococcus iniae* and other aquatic bacteria from the Microbiology and Immunology culture collection at James Cook University (JCU). The strain numbers, species, original source and identifying party are listed in Table 3.1. Several bacterial isolates were included in the library for this study because they were misidentified as *S. iniae* until PCR assays were undertaken for proper identification.

## Table 3.1 Trial bacterial isolates

| Strain | Original Label | Species | Source | Identified |
|---|---|---|---|---|
| S1 | 0-43154-1 | *Streptococcus iniae* | *Lates calcarifer* gill | A. Thomas, QLD DPI |
| S2 | 0-43154-2 | *Streptococcus iniae* | *Lates calcarifer* gill | A. Thomas, QLD DPI |
| S3 | 98-47701-1 | *Streptococcus iniae* | Moribund *Lates calcarifer* kidney | A. Thomas, QLD DPI |
| S4 | 98-47701-9* | *Streptococcus iniae* | Moribund *Lates calcarifer* | A. Thomas, QLD DPI |
| S5 | 0-49386-3** | *Streptococcus iniae* | Unknown | A. Thomas, QLD DPI |
| S6 | 98-49835-1 | *Streptococcus iniae* | Moribund *Lates calcarifer* kidney | A. Thomas, QLD DPI |
| S7 | 98-49835-2 | *Streptococcus iniae* | Moribund *Lates calcarifer* heart | A. Thomas, QLD DPI |
| S8 | 98-51343-1 | *Streptococcus iniae* | *Lates calcarifer* | A. Thomas, QLD DPI |
| S9 | 98-51343-1 | *Streptococcus iniae* | *Lates calcarifer* | A. Thomas, QLD DPI |
| S10 | 1-41291 | *Streptococcus iniae* | *Lates calcarifer* | A. Thomas, QLD DPI |
| S11 | 1-43034-2 | *Streptococcus iniae* | *Lates calcarifer* heart | A. Thomas, QLD DPI |
| S12 | 94-40210-4** | *Streptococcus iniae* | Unknown | A. Thomas, QLD DPI |
| S13 | 95-40786-6 | *Streptococcus iniae* | Moribund *Lates calcarifer* | A. Thomas, QLD DPI |
| S14 | 95-47554-1 | *Streptococcus iniae* | *Lates calcarifer* heart | A. Thomas, QLD DPI |
| S15 | 98-43935-11* | *Streptococcus iniae* | *Lates calcarifer* | A. Thomas, QLD DPI |
| S16 | ATCC 29177 | *Streptococcus iniae* | *Iniae geoffrensis* | JCU |
| S17 | | *Streptococcus iniae* | Unknown | JCU |
| S18 | | *Streptococcus iniae* | Moribund *Lates calcarifer* | JCU |
| S19 | | *Streptococcus iniae* | Moribund *Lates calcarifer* | JCU |
| S20 | K6 | *Streptococcus iniae* | Unknown | JCU |
| S22 | | *Streptococcus iniae* | Unknown | JCU |
| S23 | | *Streptococcus iniae* | Water sample | JCU |
| S24 | | *Streptococcus iniae* | Water sample | JCU |
| S25 | | *Streptococcus iniae* | Unknown | JCU |
| S26 | | *Streptococcus iniae* | Unknown | JCU |
| S27 | | *Streptococcus iniae* | Water sample | JCU |
| S28 | | *Streptococcus iniae* | Moribund *Lates calcarifer* | JCU |
| S29 | | *Streptococcus iniae* | Moribund *Lates calcarifer* | JCU |
| S30 | | *Streptococcus iniae* | Moribund *Lates calcarifer* | JCU |
| S31 | | *Streptococcus iniae* | Moribund *Lates calcarifer* | JCU |
| S32 | | *Streptococcus iniae* | Unknown | JCU |
| S33 | | *Streptococcus iniae* | Unknown | JCU |
| S34 | | *Streptococcus iniae* | Healthy *Lates calcarifer* | JCU |
| S35 | | *Streptococcus iniae* | Unknown | JCU |
| S36 | | *Streptococcus iniae* | Moribund *Lates calcarifer* | JCU |
| S36a | | *Streptococcus iniae* | Healthy *Lates calcarifer* | JCU |
| S37 | | *Streptococcus iniae* | Healthy *Lates calcarifer* | JCU |
| S38 | | *Streptococcus iniae* | Moribund *Lates calcarifer* | JCU |
| S39 | | *Streptococcus iniae* | Moribund *Lates calcarifer* | JCU |
| S40 | | *Streptococcus iniae* | Moribund *O. mykiss* (Israel) | JCU |
| S41 | AS-04-1503#7 | *Streptococcus iniae* | *Lates calcarifer* | N. Buller, WA DA |
| S42 | AS-04-1524#1 | *Streptococcus iniae* | *Lates calcarifer* heart | N. Buller, WA DA |
| S43 | AS-04-0018#1 | *Streptococcus iniae* | *Lates calcarifer* liver | N. Buller, WA DA |
| S44 | AS-04-0006#1 | *Streptococcus iniae* | *Lates calcarifer* spleen | N. Buller, WA DA |
| S45 | AS-01-2199#1 | *Streptococcus iniae* | Flying Fox Fish & guppies | N. Buller, WA DA |
| S46 | SPS#128(CCUG#47771) | *Streptococcus iniae* | Farmed fish | T.Smithyman, SPH |
| S47 | SPS#130(CCUG#47769) | *Streptococcus iniae* | Human infection | T.Smithyman, SPH |
| S48 | SPS#129(CCUG#27303) | *Streptococcus iniae* | *Iniae geoffrensis* | T.Smithyman, SPH |
| S56 | | *Streptococcus iniae* | Subcultured from S42 | JCU |
| S58 | | *Streptococcus dysgalactiae* | Moribund *Lates calcarifer* | JCU |
| L49 | | *Lactococcus lactis* ssp. *lactis* | *Oxyeleotris lineolatus* | JCU |
| L51 | | *Lactococcus lactis* ssp. *lactis* | Moribund *Lates calcarifer* | JCU |
| L52 | | *Lactococcus lactis* ssp. *lactis* | Moribund *Oxyeleotris lineolatus* | JCU |
| L53 | | *Lactococcus lactis* ssp. *lactis* | Moribund *Oxyeleotris lineolatus* | JCU |
| L57 | | *Lactococcus lactis* ssp. *lactis* | S42 contaminant (likely L49) | JCU |
| E50 | | *Enterococcus faecalis* | Moribund *Lates calcarifer* | JCU |
| Cf54 | | *Citrobacter freundii* | Water sample | JCU |
| Cf55 | | *Citrobacter freundii* | Water sample | JCU |
| C21 | | *Carnobacterium divergens* | Water sample | JCU |

QLD DPI= Queensland Department of Primary Industries; JCU= James Cook University; WA DA= Western Australia Department of Agriculture; SPH= Special Phage Holdings (Sydney, Australia)
*Altered DPI accession number. Changes were made for unknown reasons by previous researchers; it is likely these isolates are from the correct animal accession but the final number has been altered (ie. -9 and -11).
**Invalid DPI accession number.

### 3.1.2 Preservation and recovery of bacterial isolates

Monocultures of bacterial isolates were preserved at -80°C using the Microbank porous bead system (Pro-Lab Diagnostics, Canada). To recover an isolate, a single bead was removed aseptically from the frozen vial into broth culture media (see below) brought to room temperature.

### 3.1.3 Culture media

All bacterial isolates used in this study were cultured using heart infusion broth (HIB; Oxoid, Australia) or heart infusion agar (HIA), which was made by the addition of 0.75% (unless otherwise stated) technical agar (Oxoid, Australia) to HIB prior to autoclaving the media.

### 3.1.4 Preparation of broth cultures

HIB cultures were inoculated using single Microbank beads of the preserved isolates. Inoculated broths were placed into aerobic incubation at 28°C with rotation at 115 rpm.

### 3.1.5 Bacterial lawn technique

Bacterial lawns were made by pipetting 1 ml of fresh broth culture (Section 3.1.4) onto a sterile HIA plate and swirling the plate to ensure complete coverage of the agar with culture. Excess culture was removed from the plate with a pipette and lawns were allowed to dry for 30 minutes at room temperature (uncovered in a PC2 hood) prior to use.

## 3.2 Polymerase chain reaction identification of bacterial isolates

Identification of bacterial isolates was confirmed with a PCR assay for the lactate oxidase (*lctO*) gene of *S. iniae* using the primer combination LOX-1/LOX-2 (Mata *et*

*al.*, 2004a), and a16S rDNA PCR assay using universal primers 27F/1492R (Appendix 2). Amplifications were carried out in a Mastercycler gradient (Eppendorf, Australia). PCR products were cleaned and sequenced by Macrogen Inc. (Korea).

### 3.3 Spectrophotometric Analysis of Bacterial Cultures

To determine the optical density of a bacterial culture, 1 ml of the culture was transferred aseptically into a disposable 10 × 4 × 45 mm cuvette (Sarstedt, Australia) and placed into a Jenway 6300 spectrophotometer (Crown Scientific, Australia) for measurement.

### 3.4 Ultracentrifugation

Ultracentrifugation steps used for the concentration of phage from culture or environmental samples were performed at 200,000 *g* for 4 hours at 4˚C in a 50.2 Ti rotor (Optima L-90K Ultracentrifuge, Beckman Coulter). If the samples did not fill the tubes, sterile HIB was used to fill the tubes to an appropriate level.

### 3.5 Transmission Electron Microscopy

TEM analysis of was performed by Richard Webb (University of Queensland, Brisbane). A drop of phage suspension was placed onto a glow-discharged Formvar-coated 200 mesh copper grid and allowed to adsorb for two minutes. Excess liquid was removed with filter paper and a drop of 1% uranyl acetate was added, given time to wet fully and then was blotted dry. Samples were observed in a JEOL JEM1010 transmission electron microscope operated at 80kV and images were collected on a SIS Megaview III digital camera.

### 3.6 Phage Nomenclature

Discovered phages were named following the phage nomenclature scheme of Kropinski *et al.* (2009). The preface of the name identifies the phage as a bacterial virus and specifies the host and virus family (ie. vB_SinS; vB for bacterial virus, Sin for *S. iniae*, S for Siphoviridae). The second part of the name is a number that provides the specific laboratory designation for that phage.

### 3.7 Biosafety Approval

The James Cook University biosafety approval number for this project is MI07-14.

# CHAPTER FOUR

# INDUCTION OF LYSOGENIC BACTERIOPHAGES FROM *STREPTOCOCCUS INIAE*

## 4.1 Introduction

While both lytic and lysogenic phages are specific for certain bacteria, they differ in their action on the host cell following attachment and insertion of their nucleic acid. Lytic phages immediately begin viral replication inside the cell that results in cell lysis and the release of phage progeny into the environment. Lysogenic phages integrate their viral genome into the host bacterial genome, creating a prophage, which can remain and replicate with the bacterial genome for many generations. Lysogenic phages can eventually become lytic, often when the host cell is under stress (Douglas, 1975). Prophages may cause undesired phenotypic changes including the introduction of virulence factors to bacteria (Barksdale and Arden, 1974; Brussow *et al.*, 2004; Carlton *et al.*, 2005). Lysogenic phages also provide host bacterial immunity to infection by related lytic phages (termed superinfection immunity) (Barksdale and Arden, 1974; Jiang and Paul, 1998; Day, 2004). Lytic phages do not possess the genetic factors required for genomic integration, and thus will never produce a prophage in a bacterium (Carlton *et al.*, 2005). For this reason, it is safer to employ lytic phages in therapy against bacteria to avoid problems associated with lysogenic integration (Carlton *et al.*, 2005).

Lytic phages used in previous therapy trials against aquatic pathogens were originally sourced from the environment or animals where a constant population of the target bacteria exists (Nakai *et al.*, 1999; Park *et al.*, 2000; Nakai and Park, 2002; Crothers-Stomps *et al.*, 2009). Most researchers found it necessary to enrich environmental samples in target bacteria to increase the concentration of phages in the samples for further study (Park *et al.*, 2000; Crothers-Stomps *et al.*, 2009). The selection of susceptible target strains for enrichment is essential, and lysogenic bacterial strains

should be excluded due to superinfection immunity. No previous studies have identified strains of *S. iniae* that carry prophage genes, making the selection of enrichment strains for this bacteria misguided at best.

Lysogenic phages can be genetically modified for use in research and therapy of bacterial pathogens, making their discovery and characterisation even more valuable in the search for a better treatment of *S. iniae*. Specific phages can be used as delivery vehicles for DNA encoded vaccines or antibacterial treatments and also as diagnostic tools, as in phage typing (Clark and March, 2006; Serwer *et al.*, 2007). The phage delivery systems trialled previously have been found to target host bacteria at a high frequency (Westwater *et al.*, 2003). There is also potential for genetic modification to a lysogenic phage to render it permanently lytic by the removal of repressor and integration genes.

To identify lysogeny in a bacterium, it is necessary to induce lysogenic phages into the lytic cycle, which will lead to death of the host cell. While not all lysogenic phages are inducible (Barksdale and Arden, 1974), induction of most phages can be achieved by a range of environmental stressors including irradiation, dessication and nutrient starvation (Day, 2004). Mitomycin C (MMC) induction is a good method for identifying lysogenic bacterial strains because this DNA damaging agent produces more reliable changes in culture optical density than ultraviolet radiation (Loessner *et al.*, 1991). Latent periods for induced lysogenic phage have been measured between 1 and 4 hours (Davidson *et al.*, 1990).

This chapter aims to identify lysogenic strains of *S. iniae*, and to briefly characterise the lysogenic phages found to be associated with the bacteria. This study is necessary for the proper exclusion of lysogenic strains of *S. iniae* from enrichments for lytic phage, and a valuable potential exists for the identified lysogenic phages to be genetically engineered, rendering them useful for phage therapy or lethal agent delivery. This research also opens the door to further studies concerning possible phage-mediated virulence in this fish pathogen.

## 4.2 Materials and Methods

### 4.2.1 Antagonism screening

Cross-spotting assays were performed initially to screen for direct and indirect antagonism (caused by bacteriocin or bacteriophage activity) between *S. iniae* isolates in the JCU collection. To test for direct antagonism, supernatants from each strain were spotted onto lawns of each strain. Supernatants were obtained from high optical density (~40h growth) cultures of each *S. iniae* isolate (Table 3.1) centrifuged at 4300 *g* for ten minutes. To test for indirect antagonism, filtrates were produced by filtering a portion of each supernatant through 0.45µm syringe filters. Supernatants and filtrates were stored at 4°C until used. Bacterial lawns were made from each *S. iniae* isolate (Section 3.1.5) and 5 µl of each supernatant and filtrate was spotted on each lawn, along with a control spot of HIB. The supernatants and filtrates were also spotted onto control HIA plates with no bacterial lawns to test for bacterial growth in the samples. Spotted lawns were incubated aerobically at 28˚C and checked for clearance at 24, 48 and 72 hours.

### 4.2.2 Mitomycin C assays

Each *S. iniae* isolate in the collection (excluding subculture 36a) was grown aerobically in HIB overnight at 28°C, 125 rpm, diluted with HIB and grown again to an optical density of approximately 0.2 at 600 nm to ensure active growth of the cultures. Cultures were then split into two equal aliquots in sterile capped tubes, and one aliquot was treated with 250 ng $ml^{-1}$ freshly constituted mitomycin C (Sigma, Australia) to induce the lytic cycle in lysogenic strains. Incubation was continued and optical density was measured every two hours over an interval of 24 hours. An initial increase followed by a sharp reduction in optical density in the MMC-treated culture compared to the respective control was marked as indicative of the presence of a prophage in that strain. MMC inductions were repeated in triplicate on isolates showing growth patterns indicative of lysogeny to confirm the patterns observed. The effects of higher (350 ng $ml^{-1}$) or lower (150 ng $ml^{-1}$) concentrations of MMC were also examined.

### 4.2.3 Isolation and concentration of bacteriophages

To isolate the induced phages, incubation of selected samples was stopped seven hours following the addition of MMC. The samples were immediately centrifuged at 4500 *g* for five minutes, then supernatants were filtered through 0.45µm syringe filters and concentrated by ultracentrifugation (Section 3.4). Supernatants were drained immediately and the phage pellets were resuspended in 1% of original volume (300µl) SM buffer (Appendix 1.6). Concentrated phage samples were stored in 4˚C until used.

### 4.2.4 Host range determination against *S. iniae* isolates

To determine the host range of the phages within the collection of *S. iniae*, a spot-on-lawn assay was used to confirm the presence or absence of plaque formation on each *S. iniae* isolate. Bacterial lawns were made using overnight growth of the *S. iniae* isolates (Section 3.1.5, excluding subculture 36a). Concentrated phage samples were removed from storage and spotted directly onto the dried lawns in triplicate 5 µl spots. SM buffer was spotted on each lawn as a control. Spotted lawns were incubated at 28°C and checked for plaque formation within 24 hours.

### 4.2.5 Determination of phage concentration

To determine the concentration of viable phage particles (measured in plaque forming units; PFU) in the concentrated lysates with confirmed phage activity, a ten-fold serial dilution was performed for each sample using SM buffer. The dilutions were spotted in 10 µl aliquots with an SM control onto a bacterial indicator lawn. *S. iniae* S43 was chosen as the indicator strain for PFU determinations due to its susceptibility to all phages being tested. Spotted lawns were incubated at 28°C and individual plaques were counted within 24 hours.

### 4.2.6 Transmission electron microscopy

TEM analysis of concentrated induced samples was performed by Richard Webb (University of Queensland, Brisbane) (Section 3.5).

### 4.2.7 Bacteriophage DNA extraction

A modified extraction technique (Sambrook *et al.*, 1989) was used to extract phage DNA from the concentrated lysates. Prior to lysis of the phages, bacterial genomic DNA was broken down by the addition of deoxyribonuclease 1 (DNase 1; Sigma, Australia) to a final concentration of 20 µg $ml^{-1}$ and incubation for one hour at 37˚C. DNase was then denatured for 10 minutes at 75˚C for inactivation. Ribonuclease A (Sigma, Australia) was added to a final concentration of 10 µg $ml^{-1}$ and the samples were incubated for one hour at 25˚C to remove RNA. The phage heads were then lysed by the addition of EDTA, pH 8 (Appendix 1.2; final concentration 20 mM), proteinase K (Sigma, Australia; final concentration 300 µg $ml^{-1}$), and 20% SDS (Appendix 1.11; final concentration 0.5%) and incubated for 1 hour at 56˚C.

The nucleic acids were extracted using a standard phenol, phenol/chloroform and chloroform extraction. Mixing by inversion and centrifugation for ten minutes at 5000 *g* were performed for each step in the extraction. Phage DNA was purified using 0.3 M sodium acetate and ethanol washes according to Sambrook *et al.* (1989). The final product was resuspended in 50 µl nuclease-free water and stored at 4˚C until used. Nucleic acid yields were determined by $OD_{260/280}$ ratio readings using a nanophotometer (Implen, Germany). DNA extracts with low yields were freeze dried (Cryodos Freeze Dryer; Telstar) to further concentrate the nucleic acids.

### 4.2.8 Restriction digest assay

To compare the phages and estimate genome sizes, extracted DNA was digested using a standard restriction digest assay with the endonuclease *Eco*RI (Fermentas, Australia). Reactions consisted of the DNA extract (approximately 1 µg) combined with 0.5 µl *Eco*RI, 2 µl *Eco*RI Buffer (Fermentas, Australia), and sterile deionized water to make the total reaction volume 20 µl. Restriction digests were incubated for 1.5 hours at 37˚C. Digestion patterns were observed on 0.8% agarose gel stained with ethidium bromide (0.5g $ml^{-1}$) (Genesnap; Syngene, England). All samples were run with Generuler DNA ladder mix (Fermentas, Australia). Band sizes were analysed using GeneTools (SynGene, England).

## 4.3 Results

### 4.3.1 Antagonism Screening

No lysis (clearance of lawn or plaque formation) was observed from any strain tested in the indirect antagonism cross-spotting assay in which the cultures were filtered before testing on lawns. No growth was observed from the filtered supernatants on the control plates. In the direct antagonism cross-spotting assay in which cultures were not filtered, bacteria were present in each of the supernatants spotted on the control HIA plates. Lysis was observed on 4 out of 48 host lawns (8.33%), and the clearance on these lawns was associated with 19 out of 48 donor supernatants (39.58%). The most sensitive host strains were isolates S44 and S3, with lysis caused by 15 and 9 different donors, respectively (Table 4.1).

**Table 4.1** Direct antagonistic activity produced by *S. iniae* supernatants against other *S. iniae* host isolates. Filled boxes denote clearance of the host bacterial lawn by the donor supernatant. Donors and hosts showing no antagonistic activity were omitted.

**SUPERNATANT DONOR**

| HOST | S6 | S9 | S13 | S15 | S16 | S18 | S19 | S22 | S24 | S25 | S26 | S27 | S28 | S29 | S30 | S36 | S36a | S39 | S48 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| S2 | | | ■ | | | ■ | | | | | | | | ■ | | | | | |
| S3 | | ■ | | | ■ | | ■ | ■ | ■ | ■ | | | | ■ | ■ | ■ | | | |
| S14 | | | | | | | | | | | | | | | | | | | ■ |
| S44 | ■ | | | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | ■ | | ■ | ■ | |

### 4.3.2 Mitomycin C assays

MMC treated growth curves indicative of lysogeny, marked by an initial increase followed by a steep reduction in optical density between 3-7 hours following induction, were observed in 10 out of 48 *S. iniae* isolates (20.83%). Indicative growth patterns were exhibited by S2, S3, S14, S19, S27, S44, S45, S46, S47 and S48 (Figure 4.1). All other isolates exhibited non-indicative growth patterns, which showed reduced growth from the MMC treated culture relative to the control culture, but no steep reduction in optical density (Figure 4.2).

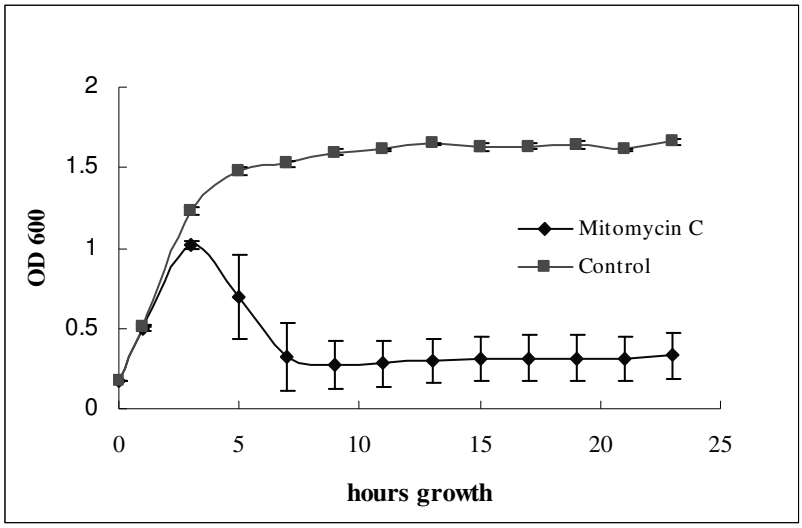


**Figure 4.1** Growth curves indicative of lysogeny measured from *S. iniae* S48, showing a steep decline in optical density of the treated culture between 3-7 hours after the addition of mitomycin C. The results are presented as means (±SD; n=3).

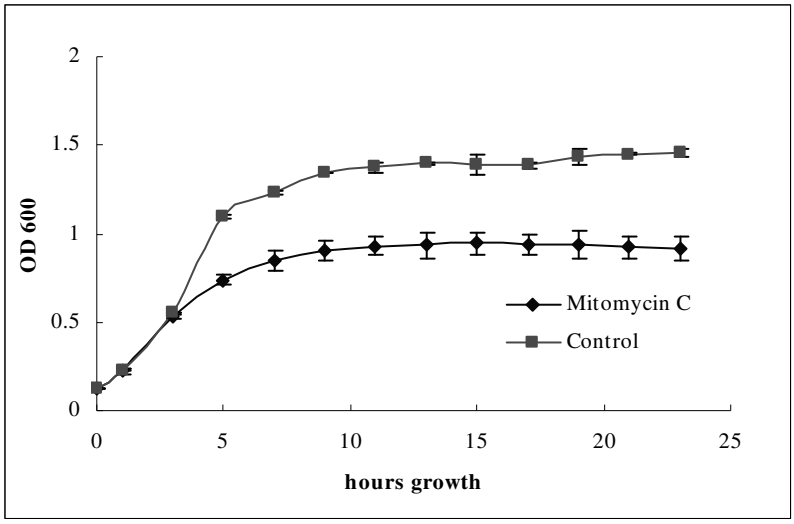


**Figure 4.2** Growth curve indicative of a non-lysogenic strain measured from *S. iniae* S12. The results are presented as means (±SD; n=3).

### 4.3.3 Phage host range against *Streptococcus iniae*

Seven of the concentrated induced samples produced individual plaques or confluent lysis on other isolates of *S. iniae* (Figure 4.3). This result confirms that 7 out of 48 isolates (14.58%) in the library harbor phages with the ability to produce lysis on other isolates. Induced samples from S2, S27 and S47 did not produce plaques on indicator lawns. Following the phage nomenclature scheme of Kropinksi *et al.* (2009) (Section 3.6), the confirmed lysogenic phages were designated with the name vB_Sin (vB: bacterial virus; Sin: the host genus and species, ***S****treptococcus* ***in****iae*), along with the number of the isolate they were induced from (ie. vB_Sin-44 is the lysogenic phage induced from *S. iniae* S44). Note: the family of virus is not included until confirmed by TEM (see discussion). Phage vB_Sin-3 produced plaques on 9.7% of *S. iniae* isolates, vB_Sin-14 and vB_Sin-19 lysed between 40-50% of the isolates, and vB_Sin-44, vB_Sin-45, vB_Sin46 and vB_Sin-48 lysed over 78% of isolates (Figure 4.4). Phage vB_Sin-45 showed the broadest host range, producing lysis on 90.9% of all *S. iniae* isolates in the JCU collection. While vB_Sin-44, vB_Sin-45, vB_Sin46 and vB_Sin-48 all displayed broad host ranges within the species, none produced lysis on the exact same selection of *S. iniae* hosts.

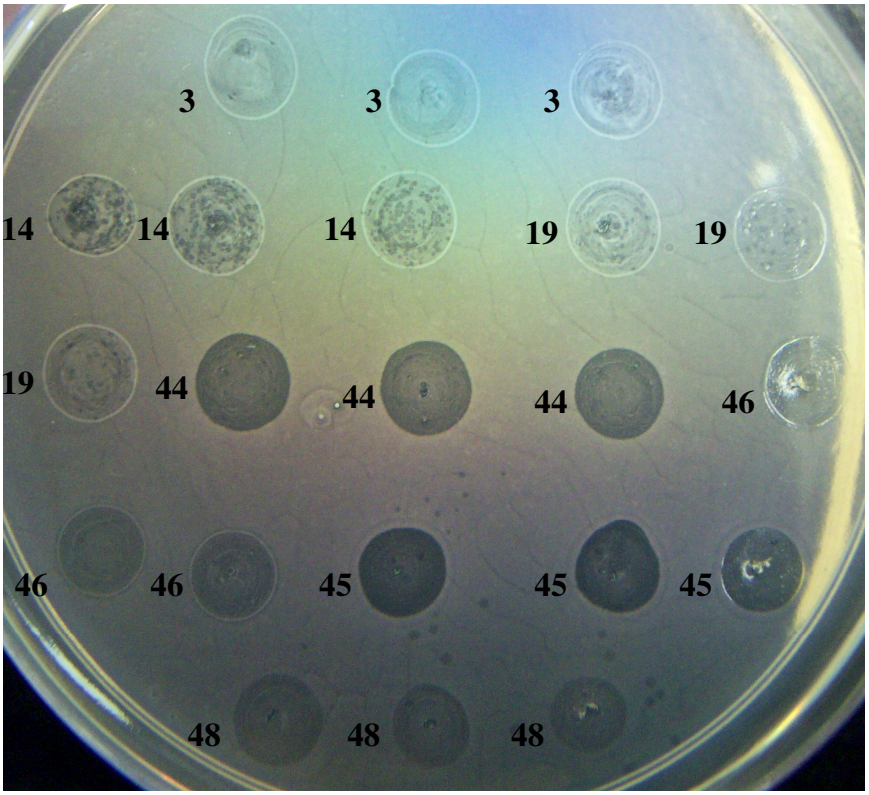


**Figure 4.3** Bacterial lawn of *S. iniae* S23 spotted with concentrated induced samples from *S. iniae* isolates S3, S14, S19, S44, S45, S46 and S48. The induced sample from S3 produced no lysis on S23, exhibiting the same spot appearance as the control (not shown). Individual plaques can be seen from phages vB_Sin-14 and vB_Sin-19 and confluent lysis was produced by phages vB_Sin-44, vB_Sin-45, vB_Sin-46 and vB_Sin-48 on this host.

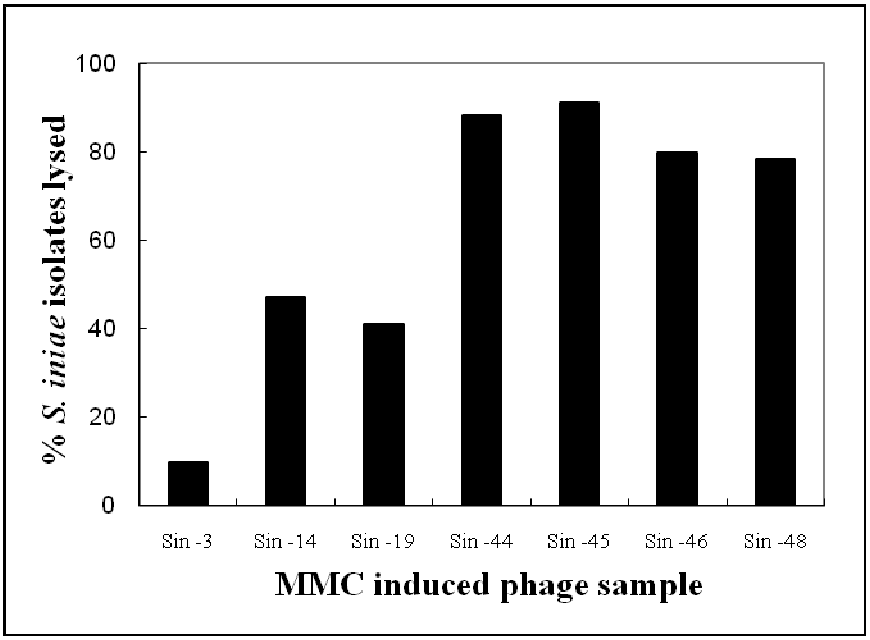


**Figure 4.4** Percentage of *S. iniae* isolates lysed by induced *S. iniae* lysogenic phages.

### 4.3.4 Phage concentrations

The concentrated induced phage samples varied in terms of how many viable phage particles were present (Table 4.2). Phage vB_Sin-19 was the least concentrated, present at $10^3$ PFU $ml^{-1}$, and phage vB_Sin-45 was present at the highest concentration of $10^{11}$ PFU $ml^{-1}$.

**Table 4.2** Plaque forming unit measurements of concentrated induced phage samples from *S. iniae*.

| Phage | Concentration (PFU $ml^{-1}$) |
|---|---|
| vB_Sin-3 | $1.3 \times 10^4$ |
| vB_Sin-14 | $4.0 \times 10^4$ |
| vB_Sin-19 | $9.0 \times 10^3$ |
| vB_Sin-44 | $1.6 \times 10^{11}$ |
| vB_Sin-45 | $4.2 \times 10^{11}$ |
| vB_Sin-46 | $1.3 \times 10^5$ |
| vB_Sin-48 | $8.0 \times 10^5$ |

### 4.3.5 Transmission electron microscope analysis

TEM analysis revealed abundant phages in samples of vB_Sin-44, vB_Sin-45, vB_Sin-46 and vB_Sin-48 (Figure 4.5). No phages were found during TEM analysis of samples vB_Sin-3, vB_Sin-14 and vB_Sin-19. The four phages photographed all exhibited long, flexible non-contractile tails and isometric heads. Base plates were visible in some intact phage particles from each positive sample. Most of the phages photographed still contained nucleic acid in the capsid head, except vB_Sin-48, in which the majority of phage particles showed empty heads. Only full capsid heads were used to calculate average head diameters (Table 4.3).

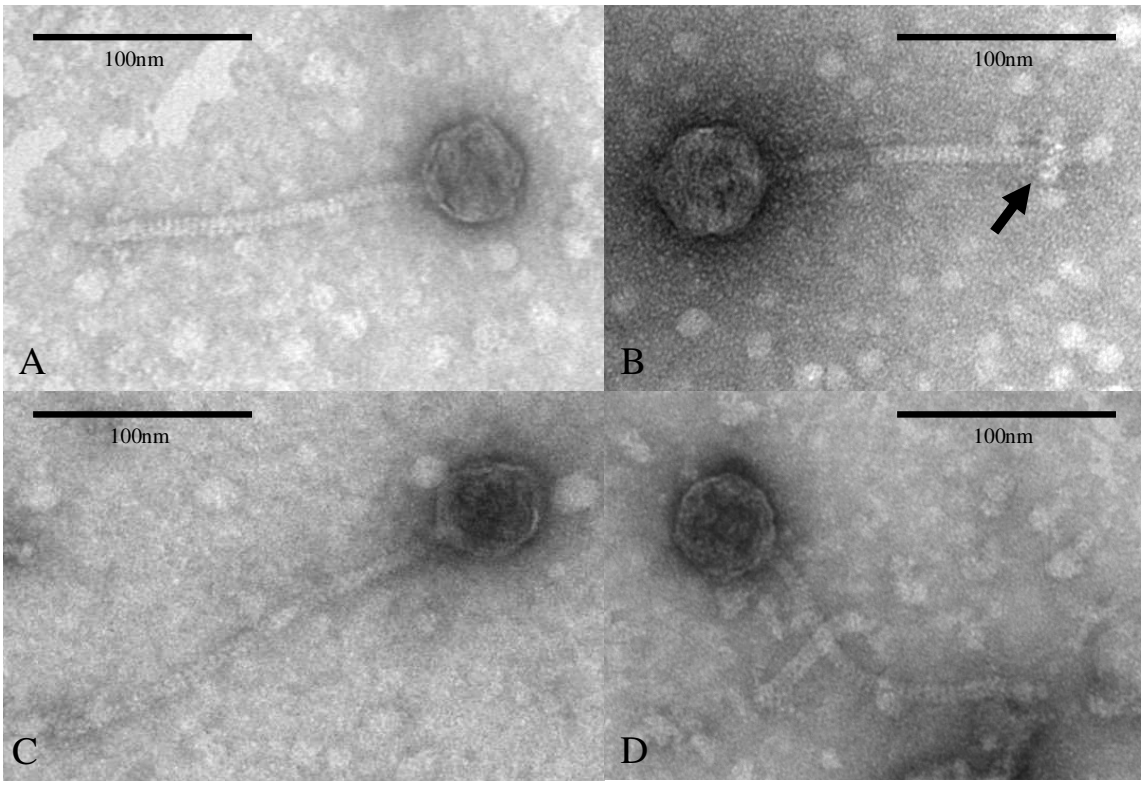


**Figure 4.5** TEM photos of lysogenic phages induced from *S. iniae*. A: vB_Sin-44; B: vB_Sin-45 with visible base plate (arrow); C: vB_Sin-46; D: vB_Sin-48. The scale bars represent 100 nm.

**Table 4.3** Measurements of phage particles derived from TEM. Average dimensions were based on a minimum of four phages.

| Phage | Head diameter (nm) | Tail length (nm) | Tail width (nm) |
|---|---|---|---|
| vB_Sin-44 | 48.35 (±1.25) | 148.63 (±9.87) | 8.29 (±0.95) |
| vB_Sin-45 | 49.42 (±1.43) | 141.48 (±5.27) | 7.62 (±0.54) |
| vB_Sin-46 | 49.37 (±2.21) | 157.93 (±12.67) | 6.93 (±0.62) |
| vB_Sin-48 | 50.79 (±5.54) | 146.77 (±7.20) | 8.67 (±1.15) |

### 4.3.6 Restriction digests of phage DNA

Phages vB_Sin-44, vB_Sin-45, vB_Sin-46 and vB_Sin-48 were digested by *Eco*RI, with multiple bands visible from each DNA sample after 0.8% gel electrophoresis (Figure 4.6). No bands were visible from vB_Sin-3, vB_Sin-14 or vB_Sin-19, despite freeze dry concentrating DNA to produce a high yield of nucleic acid in the restriction digest reactions. Phages vB_Sin-44 and vB_Sin-46 were cut in a similar pattern by *Eco*RI, with the exception of five bands that appear faintly on the digestion of vB_Sin-46 and

not vB_Sin-44. A different digestion pattern was exhibited by both vB_Sin-45 and vB_Sin-48, with vB_Sin-45 displaying only two bands not visible in the digest of vB_Sin-48. The phages in each similar pair displaying the most bands were used to calculate genome sizes. The estimated genome sizes deduced from the restriction digest patterns are 28.5 kbp and 66 kbp for vB_Sin-45 and vB_Sin-46, respectively (Table 4.4).

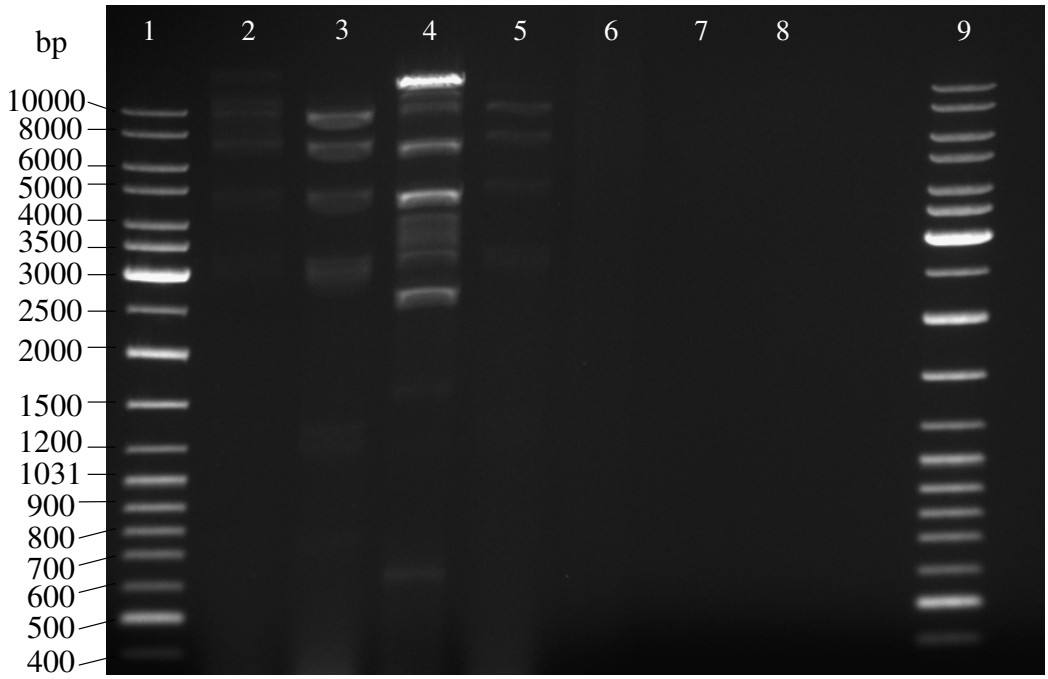


**Figure 4.6** *Eco*RI restriction digest of lysogenic phage genomic DNA. Lanes 1,9: molecular weight standards; lane 2: vB_Sin-44; lane 3: vB_Sin-45; lane 4: vB_Sin-46; lane 5: vB_Sin-48; lane 6: vB_Sin-3; lane 7: vB_Sin-14; lane 8: vB_Sin-19.

**Table 4.4** Band sizes of *Eco*RI digested lysogenic phage genomic DNA derived from GeneTools analysis.

| Phage | vB_Sin-44 | vB_Sin-45 | vB_Sin-46 | vB_Sin-48 |
|---|---|---|---|---|
| Number of visible bands | 6 | 7 | 11 | 4 |
| Band sizes (bp) | 14869.10 | 9755.11 | 14149.76 | 10772.17 |
| | 11180.35 | 7368.74 | 12500.00 | 8099.79 |
| | 10124.74 | 4885.90 | 10772.17 | 5347.39 |
| | 7444.84 | 3102.24 | 7368.74 | 3251.25 |
| | 4923.64 | 1345.69 | 4923.64 | |
| | 3144.10 | 1229.30 | 4253.96 | |
| | | 756.26 | 3825.86 | |
| | | | 3430.33 | |
| | | | 2782.00 | |
| | | | 1650.96 | |
| | | | 638.89 | |
| Approximate genome size (kbp)* | 51.69 | 28.44 | 66.30 | 27.47 |

*estimated based on visible bands

## 4.4 Discussion

This report is the first known description of lysogenic phages associated with *S. iniae*. Lysogenic phages induced from seven isolates of the bacteria had the ability to produce lysis on indicator lawns, confirming inducible prophage carriage by 14.58% of *S. iniae* isolates in this study. This rate of lysogeny is lower than rates observed in many previous studies on other bacteria. Lysogeny rates of 25% to 62% were reported in marine bacteria (Jiang and Paul, 1998), and a study on lactic acid streptococci found 43% of these strains to be lysogenic (Reyrolle *et al.*, 1982). Rates of prophage carriage can vary significantly depending on the bacterial species, origin of isolates and the assay used to determine the presence of lysogeny (Ramirez *et al.*, 1999). It is also worth noting that many of the isolates used in this study are more than 15 years old, and storage and subculturing of these isolates could have led to a loss or decay of prophage genes over time.

The assays used in this study, including mitomycin C inductions, plaque formation assays, TEM analysis and gel electrophoresis of restriction digests, all have limitations that may lead to an underestimation of the number of lysogenic bacterial strains in a collection. While MMC is considered the optimal inducing agent, it does not induce all lysogenic phages (Barksdale and Arden, 1974; Reyrolle *et al.*, 1982; Loessner *et al.*, 1991). Failure of MMC to induce every prophage from the *S. iniae* isolates screened in this study would have immediately lead to an underestimation of the number of lysogenic strains. An assay that depends on indicator strains probably also underestimates the prevalence of lysogeny in a population of bacteria (Ramirez *et al.*, 1999). Plaque assays depend on the host strain being susceptible to a particular phage, and often widespread lysogeny in many bacterial species makes finding suitable indicator strains difficult (Davidson *et al.*, 1990). However, considering the process of superinfection immunity, the high number of phage-sensitive *S. iniae* indicator hosts found in the plaque assay supports the low percentage of lysogeny observed in the collection. TEM and restriction digests will most likely return negative results if the concentration of induced phage in a sample is very low, as observed with some samples in this study. Used alone, these methods would easily lead to an underestimation of

prophage carriage rates, but together all of these techniques can be used to confirm lysogeny in bacteria.

PCR assays can be developed if a sequenced phage genome is available for that specific species and/or serotype. DNA probes for prophage have also been found during sequencing projects initially unrelated to phage. Ramirez et al (1999) observed 76% of *Streptococcus pneumoniae* strains to contain multiple *lyt*A-hybridizing fragments (a DNA probe specific for the pneumococcal autolysin gene), and found these excess genes representative of prophage carriage, though not all of these strains were inducible by MMC. PCR assays can overestimate lysogenic strains due to detection of defective prophages (Ramirez *et al.*, 1999). Another disadvantage of PCR is the detection limit; sensitivity of current systems can be so low as to require $10^7$ PFU $ml^{-1}$ (Labrie and Moineau, 2000). Also, the probed gene must be only found in prophages and not in the genomes of related lytic phages to accurately screen for the presence of lysogeny (Barksdale and Arden, 1974). If an appropriate gene probe for *S. iniae* prophages could be identified, it would be a good screening tool for lysogeny in large libraries of the bacteria.

The cross-spotting assays trialled in this study did not independently serve as reliable guides as to which strains could potentially carry prophages. It was primarily assumed that the donors causing lysis on the hosts were lysogenic, and that the sensitive hosts were free from prophage, and therefore free of lysogenic immunity. The MMC inductions indicated that quite the opposite was true. The sensitive hosts, isolates S2, S3, S14 and S44, all displayed growth patterns indicative of lysogeny in the MMC assay. However, none of the supernatant donors associated with antagonistic activity on these hosts were found to be lysogenic. It appears that direct bacterial contact induced the lytic cycle in the prophage contained in these lysogenic host lawn isolates during the cross-spotting assay. This hypothesis is supported for isolates S3, S14 and S44, which were confirmed to contain lysogenic phage, but no lysis was observed from concentrated induced samples of isolate S2. It is possible that S2 may contain a prophage that is not induced by MMC.

It is also interesting that the other six isolates with MMC curves indicative of lysogeny did not produce any antagonistic activity in the cross-spotting assays. Perhaps the prophages from isolates displaying antagonism following contact with other bacteria are more easily induced than the other prophages, which required MMC damage to induce the lytic cycle. The cross-spotting assay supported some of the findings of the MMC assay, but on its own would have lead to an underestimation of the number of lysogenic strains in the collection, or led the investigation in the wrong direction altogether.

Pseudolysogenic activity cannot be ruled out for the phages isolated during this study, though there is more evidence to suggest that they are truly lysogenic. Spontaneous release or leakage of phage particles is suggestive of pseudolysogeny (Barksdale and Arden, 1974), and there was no evidence of lytic activity from the donors during the cross-spotting assay that were later found to contain phage. Induction of these phages was also repeatable over several months of subculture (data not shown), indicating long term carriage of the viral genes, which is more common of prophage (Barksdale and Arden, 1974). Sequencing of the genomes of these phages and the bacterial genomes of the host isolates can confirm the lysogenic nature of these viruses.

Four of the lysogenic phages isolated in this study were sufficiently concentrated following ultracentrifugation ($10^5$ to $10^{11}$ PFU $ml^{-1}$) to produce positive TEM and restriction digest results. TEM analysis of phages vB_Sin-44, vB_Sin-45, vB_Sin-46 and vB_Sin-48 showed that all were tailed phages, placing them in the order *Caudovirales*. Each phage displayed an isometric head approximately 50 nm in diameter and a flexible, non-contractile tail measuring 140-160 nm in length and 7-9 nm in width, consistent with morphology of the family *Siphoviridae* (Ackermann, 2001; ICTV, 2005). *Siphoviridae* comprise the majority (60.8%) of the order *Caudovirales*, and are also the most common type of phage associated with streptococci (Ackermann, 2001). The estimated genome sizes of 28.5 kbp and 66 kbp from the two similar restriction digest patterns fit into the range of published genome sizes for *Siphoviridae* phage (ICTV, 2005). This method of calculation can underestimate phage genome size (Davidson *et al.*, 1990), and the genomes should be sequenced for confirmation.

According to Kropinksi *et al.* (2009), these four phage have an 'S' added to their names to designate the virus familial relationship (ie. vB_SinS-44).
Phages can account for many differences between strains of bacteria due to horizontal gene exchange and other effects on the bacterial genome (Brussow *et al.*, 2004). The restriction digest results have led to a hypothesis that phages vB_SinS-44 and vB_SinS-46 have similar genomes, and vB_SinS-45 and vB_SinS-48 have similar genomes different to the former pair. *S. iniae* S44 was isolated from a barramundi (*Lates calcarifer*) during a mortality event on a farm in Western Australia, and S46 was isolated from farmed fish in the United States (Table 3.1). Similarly, S45 and S48 have different origins, being isolated from quarantined fish in Western Australia and from an Amazon freshwater dolphin (*Iniae geoffrensis*), respectively. Another interesting observation is that isolate S48 is one of two type strains in the *S. iniae* collection used in this study (S48=ATCC 29178; S16=ATCC 29177), and was found to contain prophage, while the other type strain showed no results indicative of lysogeny.

The low percentage of lysogeny found in this study does not necessarily correlate to the absence of prophage genes in more (or most) *S. iniae* isolates. Many prophages are no longer inducible due to mutational decay, which can occur faster than strain differentiation within a bacterial species (Brussow *et al.*, 2004). A large proportion of prophages identified in existing bacterial genome sequences appear to be defective, and while the gene set required for induction into a reproductive cycle has been lost, they may still contain functional genes (Casjens, 2003). A defective prophage may still provide their host with superinfection immunity, which means detection assays dependent on fully inducible phages may not help researchers exclude all strains protected from lytic phage from environmental enrichment cultures.

Phages vB_SinS-44, vB_SinS-45, vB_SinS-46 and vB_SinS-48 displayed broad, but distinctive host ranges within *S. iniae*, suggesting that each phage is in fact unique. The high titres of viable phage particles following induction relative to the other isolated phages imply a large burst size by these phages. However, each phage produced different plaque sizes and concentrations depending on the indicator strain, similar to

observations made in previous studies (Reyrolle *et al.*, 1982). Regardless, the phage titre of $4.2 \times 10^{11}$ PFU $ml^{-1}$ is high following induction and ultracentrifugation. The low concentrations of vB_Sin-3, vB_Sin-14 and vB_Sin-19 suggest that these phages may be more stable in the bacterial genome, and therefore not as readily induced by MMC, as in some other phages that only show a slight increase in phage particles following induction (Barksdale and Arden, 1974). Another possibility is that isolates S3, S14 and S19 harbor multiple prophages. Low phage titres (in the same range measured from these isolates) are often measured from spontaneous induction of strains with different sets of prophages (Brussow *et al.*, 2004). Lower levels of induction in strains carrying multiple prophages could be attributed to a fitness advantage over strains carrying no prophage, and also those with fewer prophages than the bacteria in question (Bossi *et al.*, 2003). It is possible that multiple prophages carried by the same strain may reduce their burst size because their combined presence reduces the need for each to compete individually.

The host ranges observed from the phages present at lower concentrations were smaller than those of the other phages, but concentration should not affect host range. Due to the triplicate spots added to each host bacterial strain, even the least concentrated phage (vB_Sin-19; present at $10^3$ PFU $ml^{-1}$) would have been added at a rate of 150 PFU per host. It is highly unlikely that all of these phage particles would fail to produce at least one plaque on a susceptible host.

The lysogenic strains of *S. iniae* identified in this study should be excluded from enrichment cultures for environmental samples used to source naturally lytic phages. These strains will likely be resistant to attack from related lytic phages due to superinfection immunity (Jiang and Paul, 1998; Day, 2004), and therefore will be counterproductive to the enrichment process of phage multiplication. Because of the lysogenic nature of these phages, they should not be used un-modified in therapy of *S. iniae* due to the possibility of lysogenic conversion and transfer of virulence factors (Carlton *et al.*, 2005).

The broad activity spectrums and high concentrations demonstrated by phages vB_SinS-44, vB_SinS-45, vB_SinS-46 and vB_SinS-48 make them excellent candidates for future use in *S. iniae* research. The next step is to sequence the genomes of these phages to identify the genes controlling lysogeny. The prophage repressor gene, which prevents the initiation of lytic gene expression (ICTV, 2005), could potentially be removed from the lysogenic phage by genetic engineering. The expression of many genes related to viral function are silenced by repressor genes of the lysogenic genome, thus determining the stability of lysogeny (Barksdale and Arden, 1974).

Phages could be used as vectors for DNA or protein-based vaccines, or as agents of gene transfer for therapeutic purposes (Serwer *et al.*, 2007). Genetically engineered lysogenic phages can deliver DNA encoding bactericidal proteins (suicide systems) to bacteria through phagemids (Westwater *et al.*, 2003). If addiction modules, which program cell death, can be identified in the genome of *S. iniae*, toxins from these modules could be delivered to the bacterium by lysogenic phages, resulting in the death of *S. iniae* (Westwater *et al.*, 2003). A lysogenic phage with a broad activity spectrum within *S. iniae*, such as vB_SinS-44, would be excellent for phage delivery or other genetic modifications because it may not be necessary to identify the specific strain of bacteria prior to treatment.

The identified lysogenic phages could also be used in gene transfer studies to elucidate the importance of prophage association with *S. iniae* in terms of phage-mediated virulence. Prophages in other pathogenic streptococci were found to encode virulence genes and other fitness factors for the host bacteria (Ventura *et al.*, 2002). A number of the lysogenic *S. iniae* strains identified in this study were isolated from moribund animals, and lysogenic conversion could be responsible for some of the virulence factors associated with these strains. An *S. iniae* strain that showed no evidence of lysogeny in this study (eg. *S. iniae* S12) would be a good candidate for horizontal gene transfer studies using the phages.

## 4.5 Conclusion

Approximately 15% of *S. iniae* isolates in this study were confirmed to be lysogenic, suggesting low rates of lysogeny in this pathogen. This could be an underestimation due to limitations in the detection assays and also because of ongoing prophage decay. A gene probe for lysogenic phage associated with *S. iniae* would likely be a more efficient and accurate means for finding strains that should not be used in enrichment cultures. The most concentrated phages induced in this study were found to belong to the family *Siphoviridae* and genome sizes were estimated at 28 and 66 kbp. Each of these phages displayed a broad, distinctive host range within the collection of *S. iniae* isolates. These phages should be sequenced and analysed to open the door for research into phage-mediated virulence factors and genetic modification of the phages for use in therapy or prevention of *S. iniae* infections in fish.

# CHAPTER FIVE

# METHODS FOR ISOLATING LYTIC PHAGES FROM *STREPTOCOCCUS INIAE*

## 5.1 Introduction

Prevention and treatment of *Streptococcus iniae* in aquaculture remains difficult, despite the economic importance of this pathogen (Creeper and Buller, 2006). Novel antimicrobial solutions are needed to fight *S. iniae*, and bacteriophages could be an optimal new therapeutic agent, either alone or in combination with existing prevention and treatment tools.

To develop safe and effective whole phage therapy against *S. iniae*, it is necessary to find lytic phages specific for the bacteria. The probability of finding a lytic phage specific for this widespread fish pathogen should be high; there is an estimated $10^{31}$ phages on earth (Ackermann 2001). Phages are common in all habitats where bacteria live and grow, and in aquatic ecosystems, phage-like particles are more abundant than prokaryotic cells (Campbell, 2003; McAuliffe *et al.*, 2007). The high abundance of phages in nature should make it possible to isolate a substantial amount of phages able to lyse different isolates of bacteria (Bull *et al.*, 2002).

Several studies on the use of phage therapy in aquaculture have been released over the past decade. Lytic phages have been isolated and trialed in therapy against *L. garvieae* (Park *et al.*, 1997; Nakai *et al.*, 1999), *P. plecoglossicida* (Park *et al.*, 2000), *Aeromonas salmonicida* (Imbeault *et al.*, 2006), *Edwardsiella ictaluri* (Walakira *et al.*, 2008), and *Flavobacterium psychrophilum* (Stenholm *et al.*, 2008) in cultured finfish and *Vibrio harveyi* in prawn and rock lobster larvae (Vinod *et al.*, 2006; Crothers-Stomps, 2007). Lytic phages specific for these aquatic pathogens have been isolated from diseased animals, water samples from diseased ponds and outflows, and also from bacteria cultured from these sources.

Phage therapy has been trialed in Japanese flounder using phage lytic against Japanese isolates of *S. iniae* (Matsuoka *et al.*, 2007). However, because reports suggest that Asian isolates of *S. iniae* are phenotypically and genotypically different from other isolates around the world (Lau *et al.*, 2006), it is probable that phages lytic against Japanese isolates would not be viable in phage therapy against Australian isolates of the bacteria.

The aim of this study is to isolate a lytic bacteriophage specific for Australian isolates of *S. iniae* by using isolates of the bacterium to enrich environmental samples collected from fish and prawn farms where possible infection with *S. iniae* has occurred. Experiments to elucidate the *S. iniae* isolates most effective at enrichment were also performed.

## 5.2 Materials and Methods

Numerous methods to expose lytic phages specific for *S. iniae* were trialed in this study due to a lack of preliminary success using any single method. Due to time constraints, initial enrichments of environmental samples were performed before the results of the mitomycin C assay were finalised, and thus some of the bacterial isolates chosen harboured lysogenic phage(s).

### 5.2.1 Collection of samples

Environmental samples consisting of water and water/sediment were collected from four different fish farms (referred to as farms A-D) in northern Queensland and from three prawn farms (farms E-G) in the Gold Coast region of Queensland. Samples were collected from farms A and D during the summer, from farm B during the summer following a period of heavy rainfall and also during late autumn, from farm C during early summer, and from farms E, F and G during late summer. At each farm, water and sediment samples were collected from a variety of sources including grow-out ponds, raceways, system inlet and outflow areas, and settlement ponds. Farms B and C have had confirmed outbreaks of *S. iniae* in previous years. Samples collected at JCU

consisted of water from culture systems that were suspected to be contaminated with *S. iniae* from previous experiments involving the bacteria. Environmental samples were transported in coolers to maintain temperature and stored in 4˚C until used. The source and collection timing of each environmental sample is listed in Appendix 3.

### 5.2.2 Liquid medium screening

To screen for susceptible enrichment isolates, each *S. iniae* isolate in the library was combined with environmental samples to determine relative reduction in bacterial growth of the control isolate. Overnight growth of each bacterial isolate was added to a 96-well plate in 150µl aliquots. Environmental samples collected from Farm B during the summer were removed from storage and 50µl was pipetted from the top of the sample and added to duplicate wells of *S. iniae*. Control wells consisting of 150µl bacteria and 50µl PBS were made for each bacterial isolate. The plates were incubated aerobically at 28˚C with mixing overnight and the optical density of the samples was measured at 540 nm using a spectrophotometer (Multiskan EX with Genesis software; Lab-systems, Australia). Note: no 600 nm filters were available for the Multiskan spectrophotometer, and the 540 nm filter was found to provide *S. iniae* absorbance curves most similar to 600 nm (results not shown). The average optical density of the bacteria treated with an environmental sample was divided by the average optical density of the control bacteria, and all of these percentages were combined for each bacterial isolate to produce the average percent growth of each isolate treated with all environmental samples. These numbers were compared between all bacterial isolates to determine which were most affected by the environmental samples in terms of growth reduction.

### 5.2.3 Enrichments and incubations

Isolates used for enrichments were chosen from the collection of *S. iniae* strains at JCU (Table 3.1). The incubation times for the enrichment strains were varied throughout the experiments in an attempt to find optimum growth for the enrichments. Unless

otherwise stated, after the addition of the environmental sample to the enrichment isolate or cocktail, the mixtures were incubated aerobically with shaking at 115 rpm at 28˚C for the specified period. Spotted indicator lawns made during the experiments were incubated aerobically at 28˚C and checked for clearance at 24, 48 and 72 hours. Soft-top overlay plates were incubated at 28˚C and checked for clearance at 24 hours.

### 5.2.4 Enrichment in 'susceptible' isolates

Based on the results from the screening experiment, an enrichment experiment was run using the most effective environmental samples and the most *S. iniae* isolates that showed the greatest growth inhibitions. Enrichment cocktails were made from 2 ml each of overnight growth of *S. iniae* isolates S47, S28, S13 and S19. Environmental samples EB37, EB1, EB5, EB42, EB7, EB21, EB25, EB40, EA1, EA4, EA13, EA17, EA20, EJCU1 and EJCU2 were removed from storage and 0.5 ml of each was added to a separate enrichment cocktail. Following 24 and 48 hours of enrichment, 1.5 ml of each cocktail was centrifuged at 9000 *g* for 10 minutes. The supernatants were filtered through 0.45µm syringe filters and the cell-free supernatants were stored at 4˚C until used. Each cell-free supernatant was spotted onto lawns of each *S. iniae* isolate in the collection in 5µl aliquots, along with a control spot of HIB.

### 5.2.5 Enrichment in other isolates

Aliquots of 1.5 ml from each environmental sample collected from farm A were centrifuged at 2300 *g* for 10 minutes. The supernatants were added to separate enrichment cocktails made from 2 ml each of overnight growth from bacterial isolates S29, S40, S42 and L49. Following incubation for 48 hours, 1.5 ml aliquots were removed from each enrichment cocktail and centrifuged at 20000 *g* for 10 minutes. The supernatant was filtered through a 0.45 µm syringe filter and 5 µl of the resulting cell-free supernatant was spotted onto lawns of the enrichment isolates, along with a control spot of HIB.

### 5.2.6 Reduction of mechanical damage to phages

To reduce mechanical damage to phages, sterile transfer pipettes were used to transfer samples rather than pipette tips and the speed of the centrifugation step was reduced. Environmental samples EA3, EA6, EA7, EA22, EA24, EA38, EA41 and EA43 were added in 1 ml aliquots to separate enrichment cocktails made from 2 ml each of overnight growth of *S. iniae* S6, S8, S15 and S46. Following 24 hours of incubation, the enrichment cocktails were centrifuged at 1500 *g* for 10 minutes then filtered through 0.45 µm syringe filters. The resulting cell-free supernatants were stored at 4˚C until used. The supernatants were spotted onto lawns of *S. iniae* S6, S8, S15 and S46, along with an un-inoculated HIA plate to confirm the removal of viable bacteria from the supernatants. The spotting of each sample was performed with a sterile transfer pipette. Any cell-free supernatants from environmental samples showing potential activity on the incubated spotted lawns were poured onto separate HIA plates and soft-top overlays seeded with 150µl *S. iniae* S46 were added to the plates prior to incubation.

### 5.2.7 Effect of filtration on isolation of phages

Aliquots of 1.5 ml from each environmental sample collected at farm B were centrifuged at 2300 *g* for 10 minutes. The supernatants were added to separate enrichment cocktails made from 2 ml each of overnight growth from bacterial isolates S16, S42, E50 and C55. Following 48 hours of incubation, two 1.5 ml aliquots were removed from each enrichment cocktail. All aliquots were centrifuged at 9000 *g* for 10 minutes. One set of aliquots was sterilized following centrifugation by filtering each supernatant through a sterile 0.45 µm syringe filter. The other set of aliquots was left unfiltered; the supernatants from these samples were transferred to sterile microfuge tubes using sterile pipette tips. The filtered and unfiltered supernatants from the enriched environmental samples were spotted in 5 µl aliquots onto lawns of the enrichment isolates and incubated.

### 5.2.8 Repeated enrichments and concentration through ultracentrifugation and PEG

To further test environmental samples that may have shown activity in previous experiments, the samples were filtered and enriched repeatedly. Environmental samples EB1, EB5, EA1, EA4, EA13 and EJCU 1 were filtered through 0.45µm syringe filters and 2 ml of each cell-free sample was combined with 2 ml freshly grown (6 hour growth) *S. iniae* S47, S13 and S28 in separate sterile tubes. After 24 hours of incubation, the enrichment samples were centrifuged at 2800 *g* for 10 minutes to pellet the bacteria. The supernatants were added to new sterile tubes with fresh cultures of the enrichment isolates as above. This enrichment process was repeated for a total of three enrichments for each sample. Following the last enrichment, the samples were centrifuged and filtered through 0.45µm syringe filters. To concentrate any phages in the samples, the enriched cell-free supernatants were ultracentrifuged (Section 3.4). The supernatants were drained and the pellets resuspended in 300 µl SM buffer. These concentrated lysates were stored at 4˚C until used. For samples enriched in *S. iniae* S13 and S28, polyethylene glycol (PEG) was added at a concentration of 300 mg $ml^{-1}$ prior to ultracentrifugation to aid the precipitation of any bacteriophages in the sample (Seeley and Primrose, 1982). Lawns of the enrichment isolates were spotted with 5 µl of the concentrated lysates in duplicate, along with sterile SM buffer as a control. The cell-free supernatants from any environmental samples showing potential activity on the lawns were poured onto HIA plates and overlaid with soft-top agar seeded with 150µl *S. iniae* S28 prior to incubation.

### 5.2.9 Ultracentrifugation of environmental samples

Eight 50 ml environmental samples collected from farm B in autumn (EB69, EB73, EB75, EB77, EB80, EB81, EB83, EB84) were centrifuged at 4300 *g* for 15 minutes to remove bacteria and debris. The supernatants were ultracentrifuged (Section 3.4), and the pellets resuspended in 1% SM buffer and stored at 4˚C until used. Lawns of *S. iniae* S13, S19, S46 and *E. faecalis* E50 were spotted with the un-enriched concentrated

lysates using sterile transfer pipettes. The remaining lysates (approximately 250 µl each) were added to separate enrichment cocktails made from 2 ml each of bacterial isolates S13, S19, S46, S22, S30, S34, E50 and C21. After overnight enrichment, the samples were centrifuged at 4300 *g* for 15 minutes. The resulting supernatants were spotted in duplicate onto fresh lawns of the enrichment isolates.

Eight 50 ml environmental samples collected from farm A in late autumn (EA13, EA17, EA19, EA21, EA24, EA25, EA27, EA28) were centrifuged at 4300 *g* for 20 minutes to remove bacteria and debris. The supernatants were ultracentrifuged, and pellets were resuspended in 1% SM buffer and stored at 4˚C until used. The ultracentrifuged supernatants were removed from storage and 100 µl of each was added to a separate enrichment cocktail made from 2 ml each of overnight growth of bacterial isolates S8, S15, S31, S39 and L51. Following 24 hours of incubation, the enrichment samples were centrifuged at 4300 *g* for 20 minutes. The supernatants were transferred to sterile containers and the enrichment process was repeated for a total of three enrichments for each sample. Following the last enrichment, the supernatants were spotted in 7 µl aliquots onto fresh lawns of the enrichment isolates.

### 5.2.10 Enrichment with 'naïve' isolates deduced from mitomycin C tests

Following the MMC testing of the *S. iniae* isolates in the collection, the optimal enrichment isolates (those determined to be free of lysogenic phage; termed naïve) were tested with environmental samples in an attempt to isolate lytic phages specific for the bacteria. A 16 liter environmental sample collected from farm C (EC1) was enriched with 1 liter each of *S. iniae* S6, S8, S15 and S31 (grown to $O.D._{600}$ of approximately 1.50). An air stone was added to the sample to ensure mixture of the enrichment culture and the sample was left at room temperature. Following 24 hours of enrichment, twenty 50 ml aliquots were removed from the sample with a 10 ml glass pipette and centrifuged at 4300 *g* for 10 minutes at 4˚C. The supernatants were removed to 15 ml sterile tubes in 9.5 ml aliquots and 500µl chloroform was added to each tube for sterilization. The samples were mixed by inversion for approximately 30 minutes and then centrifuged at

4300 *g* for 10 minutes at 4˚C to remove the chloroform. The supernatants were removed to 10 ml sterile tubes and mixed with 10% glycerol, and the samples were stored in -20˚C until used. One sample was kept free from glycerol and was used immediately to spot in triplicate onto fresh lawns of the four enrichment isolates, along with HIB as a control.

The naïve enrichment isolates were also used to enrich environmental samples from farms D, E, F and G. Enrichment cocktails consisting of 1 ml each of *S. iniae* S6, S8, S15 and S31, 10 ml of double strength HIB and 10 ml of each respective environmental sample were incubated for 48 hours. The enrichment process was repeated using the same amounts of fresh HIB (normal strength), bacterial cultures, and the supernatants from the centrifuged (3000 *g*, 5 minutes) enrichments from the previous step were added. A total of three enrichments were performed and the final supernatants were filtered through 0.45 µm syringe filters and spotted onto lawns of the enrichment strains.

### 5.2.11 Low temperature enrichments

To test the possibility that phages lytic against *S. iniae* are only viable in environments cooler than the optimal growth temperature of the bacteria, enrichment trials were run at a reduced temperature. Four separate enrichment cultures were made, one for each enrichment strain, using 30 ml HIB, 1 ml (respectively) broth culture of *S. iniae* S6, S8, S15 and S31, and 10 ml sterile, enriched environmental sample from farm C. The enrichment cultures were incubated on an orbital shaker at 22°C. Following 48 hours of incubation, the enrichment process was repeated using the same amounts of fresh HIB, bacterial cultures, and 10 ml supernatants from the centrifuged (1500 *g*, 5 minutes) enrichments from the previous step. Six enrichments were run in total for the four bacterial enrichment strains, each incubated in 22°C for 48 hours. Following each enrichment, a small aliquot was removed from the supernatant of each centrifuged sample and stored at 4°C. During the sixth enrichment, aliquots were removed at three, six, 24 and 48 hours. At the end of the trial, each of the saved aliquots, both unfiltered and filtered through 0.45µm syringe filters, was spotted onto a lawn of the appropriate

enrichment strain along with a control spot of HIB for the observation of plaques. Control HIA plates with no bacterial lawn were also spotted with the supernatants. Spotted lawns in this experiment were incubated at 22°C and checked for clearance at 24, 48 and 72 hours.

### 5.2.12 Control experiments

Four phage samples with reported lytic activity against *S. iniae* were obtained from Special Phage Holdings (SPH, Sydney, Australia) in order to test the enrichment and assay methods being trialed in this study. Lawns of *S. iniae* S1, S2, S3, S5, S6, S7, S8, S11, S14, S15, S16, S24, S28, S31, S32, S33, S37, S38, S39, S41, S42, S43 and S46 were spotted with 5 µl aliquots of the unenriched phage samples. Spotted lawns in this experiment were incubated at low temperature (22°C) and checked for clearance at 24, 48 and 72 hours.

Enrichment cocktails consisting of 1 ml each of *S. iniae* S6, S8, S15 and S31, 10 ml HIB and 100 µl of each SPH phage sample were incubated for 48 hours. The enrichment process was repeated using the same amounts of fresh HIB, bacterial cultures, and 2 ml supernatants from the centrifuged (3000 *g*, 5 minutes) enrichments from the previous step. The same steps were repeated for a total of three enrichments, and the final supernatants were filtered through 0.45 µm syringe filters and spotted onto lawns of the enrichment strains.

One of the SPH phage samples (150 µl) was added to a single enrichment of 1 ml each of *S. iniae* S6, S8, S15 and S31, and 3 ml HIB and incubated aerobically with mixing at 28˚C overnight. The culture was then centrifuged at 4500 *g* for five minutes and filtered through a 0.45 µm syringe filter. This lysate was spotted onto lawns of the enrichment strains in 5 µl aliquots. The remaining lysate was ultracentrifuged, and the pellet was resuspended in 1% SM buffer. This sample was sent for analysis by TEM (Section 3.5). The same phage sample was enriched again, but this time in eight enrichment strains including *S. iniae* S6, S8, S14, S15, S28, S31, S34, and S46. The phage sample (250 µl)

was added to 8 ml HIB and 2 ml each of each enrichment strain (with $OD_{600}$ approximately 0.3) in a conical flask. This enrichment was duplicated and a control enrichment containing the broth and enrichment strains only was also made. Each culture was incubated aerobically with mixing at 28˚C for 20 hours. The samples were then centrifuged at 4500g for five minutes and filtered through a 0.45µm syringe filter. The lysates were spotted (in duplicate) onto lawns of each *S. iniae* strain in the library and a control HIA plate, and incubated at 28˚C for 20 hours.

## 5.3 Results

### 5.3.1 Liquid medium screening

The growth of most isolates treated with environmental sample was reduced relative to the control, with isolate S47 experiencing the greatest average reduction (treated samples $OD_{540}$ 1.54 / control $OD_{540}$ 2.04 = 0.75; Figure 5.1). Two isolates (S42 and S44) experienced increased growth when treated with the environmental samples. The results are listed in Table 5.1 with the most affected isolates and most effective environmental samples shown first.

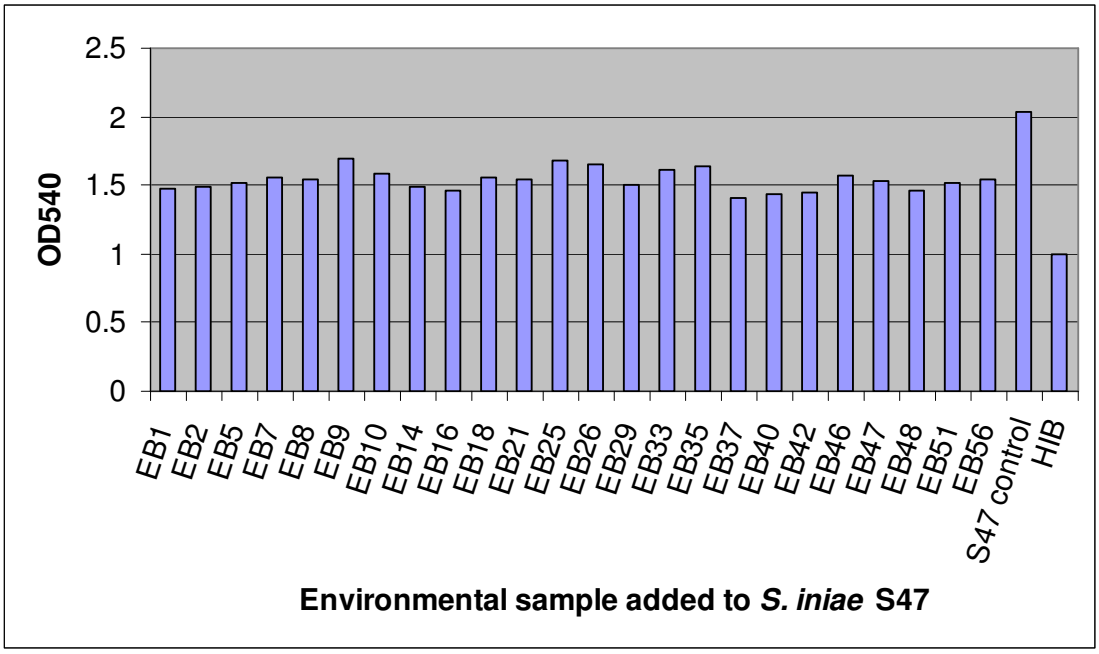


**Figure 5.1** Optical densities of *S. iniae* strain S47 when combined with environmental samples from Farm B.

**Table 5.1** Average growth percentages of each bacterial isolate when treated with Farm B environmental samples in the liquid media screen.

| ***S. iniae* isolate** | **Average % growth of isolate tested with all environmental samples**** |
|---|---|
| S47* | 75 |
| S13 | 78 |
| S6 | 79 |
| S28 | 79 |
| S15 | 79 |
| S19* | 79 |
| S46* | 80 |
| S8 | 81 |
| S14* | 81 |
| S9 | 82 |
| S20 | 82 |
| S4 | 83 |
| S16 | 83 |
| S34 | 83 |
| S7 | 84 |
| S12 | 84 |
| S22 | 84 |
| S26 | 84 |
| S29 | 84 |
| S30 | 84 |
| S43 | 84 |
| S17 | 85 |
| S25 | 85 |
| S27* | 85 |
| S33 | 85 |
| S39 | 85 |
| S40 | 85 |
| S31 | 86 |
| S32 | 86 |
| S36 | 86 |
| S37 | 86 |
| S48* | 86 |
| S18 | 87 |
| S24 | 87 |
| S35 | 87 |
| S38 | 87 |
| S3* | 88 |
| S10 | 89 |
| S23 | 89 |
| S45* | 89 |
| S1 | 91 |
| S2* | 91 |
| S11 | 91 |
| S41 | 92 |
| S5 | 93 |
| S56 | 93 |
| S42 | 103 |
| S44* | 105 |

*strains suspected of containing a prophage in mitomycin C assay.
**percent growth is relative to the growth of the control isolate.

### 5.3.2 Enrichment in 'susceptible' isolates

The lawn of *S. iniae* S47 appeared slightly cleared by environmental samples EB1, EB5, EA1, EA4, and EJCU2 when enriched in the susceptible isolates determined from the liquid media screening. There were no visible plaques on the lawn, but these spots appeared clearer than the other samples as well as the control spot of broth only. Continued experimentation with these samples including repeated enrichments and ultracentrifugation resulted in no further visible clearance of *S. iniae* S47.

### 5.3.3 Reduction of mechanical damage to phages

After 48 hours of incubation, the control HIA was free from growth, confirming the cell-free nature of the supernatant samples. There was a possible plaque effect on the *S. iniae* S46 lawn for environmental samples EA43 and EA38. The soft-top overlays produced for the follow-up experiment contained good growth of *S. iniae* S46, but it was difficult to confirm presence of plaques due to the lack of a successful control soft-top plate.

No other instances of visible clearance of the indicator lawns occurred during any of the other attempts to isolate lytic phages from the environmental samples, even after 72 hours. Some form of sterilization (ie. filtration through 0.45µm syringe filters or chloroform) proved to be necessary to remove the bacteria from lysates or supernatants to be spotted on indicator lawns. In each experiment where centrifugation steps were the only means of removing bacterial cells, there was growth from the samples on the control HIA plate. No growth on the spots of filtered or chloroform treated lysates occurred when spotted onto a sterile HIA plate.

### 5.3.4 Low temperature enrichments

The enrichment samples for each bacterial strain grew to an optical density of at least 2.0 measured at 600 nm during each enrichment. There was consistently a smaller layer

of growth present in the spots from the filtered supernatant than from the corresponding unfiltered supernatant, but this difference was also observed on the control plate with no bacterial lawn. The unfiltered aliquots all contained bacterial growth on the HIA control plate. No plaques were observed on any of the indicator lawns.

### 5.3.5 Control experiments

The unenriched and enriched phage samples from Special Phage Holdings failed to produce visible plaques on the indicator lawns of S6, S8, S15 and S31 trialed at 22°C and 28°C. However, phages were photographed using TEM from the concentrated sample resulting from enrichment in *S. iniae* S6, S8, S15, and S31 at 28°C. The phages isolated have a head diameter of 55 nm with tails measuring approximately 170 nm by 9 nm (Figure 5.2).

Due to the positive TEM result, the second enrichment was run using eight bacterial strains, and these lysates were spotted onto lawns of the *S. iniae* library. Very small plaques were observed on nine out of the 48 isolates, including *S. iniae* S3, S4, S7, S14, S23, S39, S41, S42, and S44. The only enrichment strain that plaques were observed on was *S. iniae* S14, and these plaques were incredibly small and difficult to see with the naked eye. The largest plaques observed were on lawns of strains that were not included in the enrichment, particularly isolate S3 (Figure 5.3).

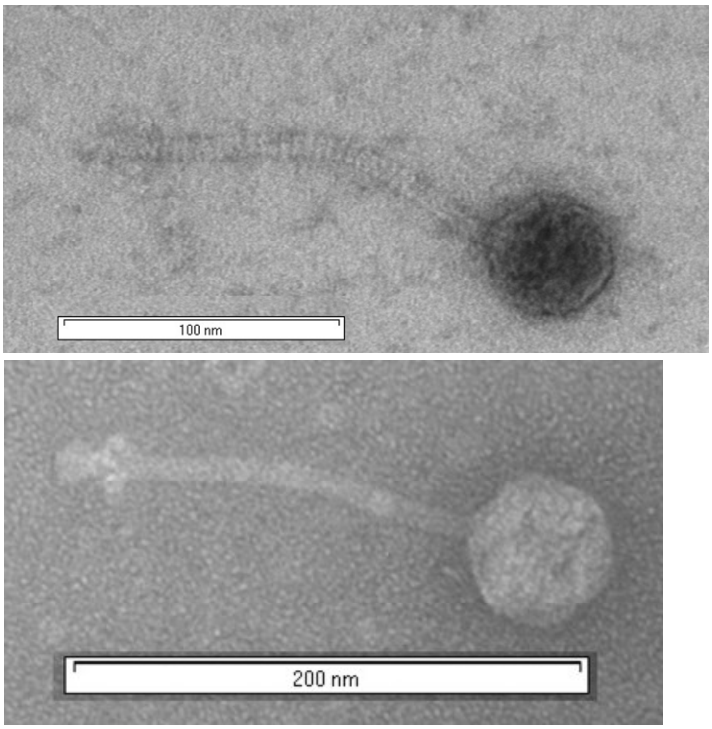


**Figure 5.2** TEM photos of Special Phage Holdings phages from sample SPH-2 enriched with *S. iniae*

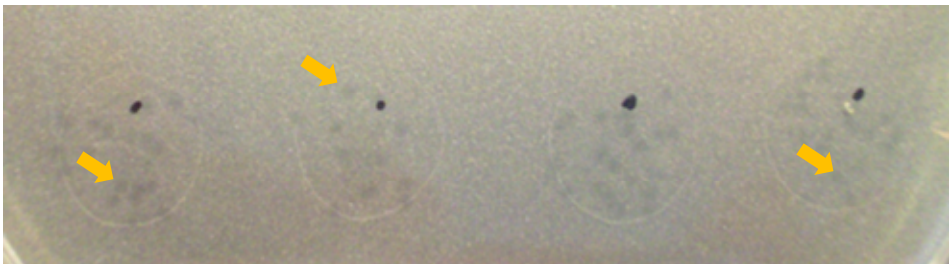

**Figure 5.3** Plaques (examples shown by arrows) caused by enriched Special Phage Holdings phages on *S. iniae* S3.

## 5.4 Discussion

The experiments performed in this study resulted in no visible plaques on indicator bacterial lawns from environmental samples. The results indicate either that 1) phages were isolated but do not create reliable, visible plaques, 2) no phages were collected in the environmental samples, or 3) collected phages were destroyed or left unviable during experimentation by damage or decay.

An issue that may have prevented the successful isolation of *S. iniae* phages in this study is the possibility that some phages do not create reliable plaques even when present in a lysate. The absence of plaques on an indicator lawn does not necessarily indicate the absence of phages in the lysate (Seeley and Primrose, 1982). Considering that the control phage sample from Special Phage Holdings created no visible plaques on indicator lawns even though phage presence in the same lysate was confirmed by TEM, it is possible that each of the experiments performed in this study may have isolated one or several phages associated with *S. iniae*. The follow-up enrichment showed that the SPH phages produced plaques on several isolates in the S. *iniae* library, but only on one strain used in the enrichment. Interestingly, three of the host lawns that the SPH phage created plaques on (isolates S3, S14 and S44) were confirmed to be lysogenic strains of *S. iniae* (Chapter 4.3.3).

The plaque assay used to confirm the isolation of lytic phages should have been supplemented with TEM photos or another assay such as restriction digest of phage nucleic acid. However, the confirmation of phage presence in a sample does not provide proof that the phages are lytic against a specific bacteria. It would be necessary to measure an increase in phage titre after enrichment in each individual bacterial isolate to conclude which isolates the phages have the ability to lyse cells.

A number of the farms sampled for phages in this study have had confirmed outbreaks of streptococcosis in the past and have undertaken vaccination programs to prevent further losses. The basic enrichment method these experiments utilized is only

successful if the sought after phage is present in the inoculum (Seeley and Primrose, 1982). If the vaccination strategies in place at the farms successfully prevented populations of *S. iniae* from multiplying to high levels, phage numbers would have also been very low in these environments as they depend on the bacteria for growth.

Mechanical damage to phage particles may have occurred during concentration or transfer of phages during the experiments performed in this study. Mechanical damage can affect the viability of phages or phage DNA, which could prevent the formation of plaques on indicator lawns. Previous studies comparing methods of concentration of *Myoviridae* and *Siphoviridae* phages found that ultracentrifugation caused less mechanical damage to phage than chemically based concentration methods (Oakey and Owens, 2000; Elliman, 2006), which is why ultracentrifugation was used most consistently as a concentration method in this study. Bacteriophages successfully isolated from other studies using methods similar to those trialed in these experiments may have been less susceptible to mechanical damage than the phages potentially present during this study.

Filtration (0.45µm and 0.22 µm) has been used to sterilize environmental samples and enrichment cocktails in several studies which successfully exposed lytic phages specific for aquatic pathogens (Nakai *et al.*, 1999; Park *et al.*, 2000; Imbeault *et al.*, 2006; Crothers-Stomps, 2007; Walakira *et al.*, 2008). Results from this study showed that when cultures were not filtered through 0.45µm syringe filters or treated with chloroform, bacteria either from the isolates used for enrichment or directly from the environmental sample remained in the supernatants spotted onto indicator lawns. With bacteria present in the spotted sample, it would have been difficult to observe plaque formation and to prove that any clearance was not created by a bacteriocin or other inhibitory interaction between bacteria, rather than a phage. These reasons supported the continued use of filters during this study, though it was understood that a reduction in phage titer may have resulted.

Filtration of samples and cultures can reduce the efficiency of phage recovery due to direct adsorption of phages onto the filter, or physical retention of phages that are associated with or adsorbed to particulate matter that are trapped on the filter (Seeley and Primrose, 1982). Phages isolated during this study were expected to belong to the family *Siphoviridae* because these phages are most frequently associated with streptococci (Ackermann, 2006b). The length range of *Siphoviridae* phages is 120-630 nm, with most having a head diameter of 60 nm and a tail length of 150 nm, equaling a normal length of 210 nm (ICTVdB_Management, 2006). As long as the phages collected during this study were shorter than 450 nm, a good number should have still passed easily through the 0.45µm filter, assuming not all were adsorbed to particulate matter or the filter itself. Pre-rinsing of the filters (using 1.5 ml HIB) was trialed in experiments toward the end of this study, but did not produce a different result from normal filtration. Filtration was completely removed from the experimental methods with the use of chloroform sterilization, but the same result of no plaque formation on the indicator lawns was found, suggesting that filtration is not to blame for the absence of evidence for phages in this study.

Decay of phages in storage prior to enrichment or assay on indicator lawns may have also prevented the formation of plaques. Numbers of naturally occurring phages from sewage samples remain stable in river water for three days at 5°C, but populations begin to decline following seven days (Mendez *et al.*, 2002). *Siphoviridae* phages specific for *L. garvieae* can also survive in unsterilized seawater for at least three days (Nakai and Park, 2002). In this study, phages were enriched and assayed promptly to prevent decay, but even a small amount of population decline could prevent the formation of a visible plaque on an indicator lawn. The large 16L sample collected from farm C was frozen down with glycerol following enrichment, and these aliquots were used for experiments in the following months because any phages in these samples should have survived longer than with simple refrigeration. Numbers of concentrated phages stored in the freezer with 5-10% glycerol have been found to remain stable for at least 50 days (Mendez *et al.*, 2002).

Enrichment was chosen as the preliminary amplification method for phage samples in this study due to the fact that it is the best technique for isolating phages for a specific host (Seeley and Primrose, 1982). Lytic phages specific against other aquatic pathogens were successfully multiplied by inoculation into enrichment cultures of host bacteria when obtained from the environment (Park *et al.*, 2000; Imbeault *et al.*, 2006; Vinod *et al.*, 2006; Crothers-Stomps *et al.*, 2009). Repeated enrichments were attempted in this study to further amplify phage populations since no phages were isolated in assays following a single enrichment. The repeated enrichments also produced no visible plaques. Considering the failure of the enrichment procedures to produce evidence of phage from these experiments, other concentration techniques were tested, including ultracentrifugation and PEG. These physical methods have both been used successfully to concentrate phages from water samples (Seeley and Primrose, 1982). The failure to isolate phages following all of these previously successful concentration and amplification methods could be due to the absence of phages in the original sample inoculum, the use of inappropriate enrichment strains, or because further concentration was required to produce visible plaques.

Enrichment strains that were not susceptible to phages in the environmental samples or were at the wrong growth stage when added to the enrichment culture would have been useless at amplifying the virions. The results from the liquid media screening of environmental samples from Farm B guided better choices for the bacterial strains being used to enrich environmental samples throughout the rest of the study. The liquid screen was a 'quick and dirty' method to choose enrichment strains based on relative reduction in optical density from a control, while the mitomycin C assay in Chapter four is based on eliminating lysogenic bacterial strains from the enrichment cocktails. Bacterial isolates containing a prophage are normally immune to infection by related lytic phages (Maloy *et al.*, 1994), and therefore would not be efficient at multiplying lytic phages in an enrichment sample. The results from the liquid screen and MMC assay were essentially combined following their completions to choose enrichment strains both affected by environmental samples and free from lysogenic phages. These isolates should have been the most efficient at amplifying phages from environmental samples.

The temperature of incubation is an important factor for phage isolation (Seeley and Primrose, 1982). There are cases in which phages grow optimally in different temperature ranges than their host bacteria. For example, another aquatic pathogen, *Lactococcus garvieae* grows in the range of 17-41°C, but phages specific for this bacteria are only active at 29°C or cooler (Nakai and Park, 2002). The optimal temperature range for growth of the *S. iniae* isolates used in this study is 26-28°C, while the full growth range is 10-37°C (Bromage, 1997). Attempts to isolate environmental phages by running enrichments and incubations at the lowered temperature of 22°C were unsuccessful, but this may have been due to the enrichment strains, prior damage to the phages, or because *S. iniae* phages do not show a temperature dependence in this range.

Further investigations should be carried out to elucidate if lytic phages active against *S. iniae* are only viable at lower temperatures. A strong association between temperatures of 25-28°C and increased mortality in barramundi has been shown (Bromage and Owens, 2009). It is possible that mortalities drop when the temperature falls below 25°C not only because this temperature is below the optimal growth range for *S. iniae*, but also because phage activity may increase in the environment at these lower temperatures.

While most techniques used in this study have been successful at isolating lytic phages against other aquatic pathogens, they failed to provide evidence of a lytic phage against *S. iniae*. This result may be due to differences in bacterial motility, spatial and seasonal distribution in the environment, and also due to the morphological types of phages targeted. Characteristics of the pathogenic aquatic bacteria and the phages isolated against them from other studies are included in Table 5.2.

**Table 5.2** Characteristics of pathogenic aquatic bacteria and associated lytic phages

| Bacteria | Gram stain | motility | spatial distribution | seasonal distribution | phage family |
|---|---|---|---|---|---|
| ***Streptococcus iniae*** (Matsuoka *et al.*, 2007) | + | non-motile | sediments (year round) | prevails in summer, 25-28°C | *Siphoviridae* |
| ***Vibrio harveyi*** (Vinod *et al.*, 2006; Crothers-Stomps, 2007) | - | motile | free living, biofilm, in association with marine animals | n/a | *Siphoviridae, Myoviridae* |
| ***Lactococcus garvieae*** (Park *et al.*, 1997; Nakai *et al.*, 1999) | + | non-motile | n/a | prevails in summer, >20°C | *Siphoviridae* |
| ***Pseudomonas plecoglossicida*** (Park *et al.*, 2000; Nakai and Park, 2002) | - | motile or non-motile | all culture environments | n/a | *Myoviridae, Podoviridae* |
| ***Aeromonas salmonicida*** (Imbeault *et al.*, 2006) | - | mostly non-motile | biofilm, sediments (year round) | 25-27°C | *Myoviridae* |
| ***Edwardsiella ictaluri*** (Walakira *et al.*, 2008) | - | motile | n/a | late spring, early fall, 18-30 °C | *Siphoviridae* |
| ***Flavobacterium psychrophilum*** (Stenholm *et al.*, 2008) | - | motile | on animals | ~18 °C | *Siphoviridae, Myoviridae, Podoviridae* |

Another difference between this study and a few of the successful isolation studies is that several phages found in those studies were isolated from diseased fish specimens or bacterial isolates from moribund fish (Park *et al.*, 1997; Park *et al.*, 2000). No fish were infected with *S. iniae* in this study, nor were any reported from any of the farms during the period of this work. It is possible that lytic phages may be more easily isolated from a fish infected with the target pathogen rather than the environment.

The lytic phages isolated against Japanese strains of *S. iniae* (Matsuoka *et al.*, 2007), may have the ability to lyse Australian strains of the pathogen, though published evidence supports the contrary. The phenotypic differences between Asian isolates and

North American isolates (Lau *et al.*, 2006) would potentially have a large impact on phage interaction with these isolates. North American isolates were found to be similar to Australian fish isolates in another study (Nawawi *et al.*, 2008). The phage infection process is dependent on interaction with cell surface receptor molecules on the host bacteria (Jensen *et al.*, 1998), and differences in haemolysis, shape and consistency of bacterial colonies like those cited from the Asian isolates (Lau *et al.*, 2006) could easily translate to changes in surface receptor molecules. Also, recently characterised phages lytic against Danish strains of *Flavobacterium psychrophilum* were not effective at lysing a type strain of this bacteria isolated in the United States, suggesting the host range of phages is limited to genetically related and co-occurring host strains (Stenholm *et al.*, 2008).

## 5.5 Conclusion

No lytic phages specific for *Streptococcus iniae* were successfully isolated in this study. It is possible that phages were isolated but did not create reliable, visible plaques, meaning that more in depth concentration techniques should be trialed. Spatial and seasonal distribution of the host bacteria, as well as farm vaccination programs may have prevented the collection of phages from environmental samples. More samples should be collected, especially at farms currently experiencing outbreaks of streptococcosis. The possibility that collected phages were destroyed or left unviable during experimentation still remains, but considering that the enrichment and assay techniques were so similar to other successful phage isolation studies, this issue is probably not the cause of failure in this study.

Professional phage researchers in Australia independently investigating lytic phages specific for *S. iniae* have also had much misfortune isolating phages for the purpose of therapy in treatment of this pathogen (Smithyman, 2009). However, it is important to continue the search for phages lytic against Australian isolates of *S. iniae* so that the efficacy of phage therapy against this pathogen in Australian aquaculture can be investigated.

# CHAPTER SIX

# CHARACTERISATION OF A BACTERIOCIN ACTIVE AGAINST *STREPTOCOCCUS INIAE*

## 6.1 Introduction

The available treatment options for *S. iniae* infections in fish need to be expanded to avoid harmful effects associated with vaccination and antibiotics. Chemical-free "green solutions" appear to be the next era of therapeutic products for preventing bacterial epizootics in fish (Abutbul *et al.*, 2004). Bacteriocins are biologically active proteins or protein complexes displaying a bactericidal mode of action against closely related species to that of the producing bacteria (Tagg *et al.*, 1976). These specific antimicrobial substances have great potential as a new therapy option for bacterial pathogens in aquaculture.

Two bacteriocin-like inhibitory substances were found during cross-spotting assays and lytic phage searches performed using the JCU *S. iniae* library. One BLIS producer was subsequently identified as *Lactococcus lactis* ssp. *lactis* and the other as *Carnobacterium divergens*. The former was chosen as the subject for an in depth characterisation and BLIS study due to its larger inhibitory spectrum on the library of *S. iniae* isolates. Only one published study on a bacteriocin produced by *Carnobacterium divergens* has included *S. iniae* in the tested indicator species (Kim and Austin, 2008). To date, there are no published studies on other bacteriocins with action against *S. iniae*, including those produced by strains of *L. lactis*.

Bacteriocins are recognized as potentially useful agents in the control of bacterial infections due to their effectiveness, non-toxicity and relatively cheap production (Brook, 1999; Reid *et al.*, 2001; Riley and Wertz, 2002). In the aquaculture industry, probiotics are anticipated as being effective replacements for antibiotics and

chemotherapeutics to fight infectious diseases, either in the form of whole cells or cell components such as bacteriocins (Hagi and Hoshino, 2009).

The majority of bacteriocins produced by Gram-positive lactic acid bacteria are produced within the genus *Lactococcus* (Guinane *et al.*, 2005). Lactococcal bacteriocins are classed as lantibiotics, or group I bacteriocins, and generally are small proteins (<5kDa) containing unusual amino acids (Parisien *et al.*, 2008). Bacteriocins produced by *L. Lactis* are generally heat resistant, active over a wide range of pH levels, and sensitive to several proteolytic enzymes including proteinase K and α-chemotrypsin (Moreno *et al.*, 2000).

Bacteriocins are generally categorized by producing strain, common resistance mechanisms, protein structure, and mode of action (Parisien *et al.*, 2008). Characterisation of a bacteriocin-like inhibitory substance is necessary for proper identification, categorization and to confirm whether the substance is a true bacteriocin.

The aims of this chapter are to characterise the discovered BLIS produced by *Lactococcus lactis* ssp. *lactis* with activity against *S. iniae* and to discuss the potential of this substance for use in treatment of *S. iniae* infections in fish.

## 6.2 Materials and Methods

### 6.2.1 Source and identification of BLIS producer

During a lytic phage isolation experiment, a lawn of *S. iniae* isolate S42 was shown not to be a monoculture; two distinct colony types had grown on the lawn, with one (designated as isolate L57) antagonizing the growth of the other (S42). Preliminary identification of both isolates was carried out by API 20 Strep and API rapid ID 32 Strep test kits (Biomerieux, United States). Complimentary tests of 45°C, 6.5% NaCl and Lancefield grouping were also performed. Identification was confirmed with PCR assays

(Section 3.2). The BLIS-producing isolate was designated as *Lactococcus lactis* ssp. *lactis* L57, and the inhibitory substance as BLIS-L57.

### 6.2.2 Isolation of BLIS-L57 from bacterial culture

To produce cell-free supernatant (cfs) containing BLIS-L57, *L. lactis* L57 was grown aerobically at 28°C for 17-18 hours, then centrifuged at 4300 *g* for 5 minutes and filtered through 0.45µm. Aliquots of cfs were stored at 4°C if not used immediately.

### 6.2.3 Confirmation of bacteriocin-like inhibitory activity

*S. iniae* S56 was used as the bacterial indicator in preliminary tests. Bacterial indicator lawns were spotted with 5µl each of overnight growth of *L. lactis* L57 and cfs from the same culture. To test for phage activity, flip plates were made by flipping the agar containing the dry indicator lawn, leaving it face down in the plate. The flipped plate was spotted with three 5µl aliquots each of overnight growth of *L. lactis* L57 and cfs from the same culture. Plates were checked for inhibition after incubation at 28°C for 24 hours. To rule out the possibility that the antagonism was caused by hydrogen peroxide production, overnight growth of *L. lactis* L57 was spotted (four 5 µl replicates) onto indicator lawns. One spotted lawn was incubated aerobically at 28°C and the other lawn was incubated in anaerobic conditions at 28°C, both for 48 hours before checking for inhibition.

### 6.2.4 Assays for antagonistic activity

#### 6.2.4.1 Spot-on-lawn assay

Antagonistic activity was detected on solid media using a spot-on-lawn assay (Hoover and Harlander, 1993). A soft agar top consisting of 5 ml 0.5% HIA was seeded with 100 µl overnight growth (approximately $10^9$ cfu) of the indicator culture and then poured onto a 0.75% HIA plate. An aliquot of 5-10 µl (depending on assay) of the

BLIS-L57 sample being tested was spotted onto the hardened soft top. Plates were made in duplicate and incubated upright for 24 hours at 28°C. Antagonism was measured as the diameter of the clear spot produced, or in some cases as positive (clearance) or negative (no clearance).

#### 6.2.4.2 Microtitre plate assay

To quantify antagonistic activity in liquid media (broths), a modified microtitre plate assay was performed. The indicator culture was grown to an optical density of approximately 0.2 measured at 540 nm, and added to dilutions of the growth media or cfs (treated or untreated) in duplicate 96-well round bottom plates. Plates were incubated aerobically for 3 hours at 28°C and the optical density was then measured at 540 nm using a Multiskan Ascent plate reader (Pathtech, Australia). Antagonistic activity was defined arbitrarily as the reciprocal of the dilution causing 50% growth inhibition relative to the control sample without cfs (AU = arbitrary units). *S. iniae* S23 was used as the indicator strain for all spot-on-lawn and microtitre plate assays.

### 6.2.5 Ampicillin test of microtitre assay

To determine the efficacy of the microtitre plate assay for indicator strain *S. iniae* S23, ampicillin was used as a test inhibitory substance. Ampicillin (0.5 μg $ml^{-1}$) was added to cultures of the indicator strain at different time intervals during the growth of the indicator beginning at an optical density of 0.49 at 540 nm. For each time test, positive and negative controls were replicated eight times by the use of a 96 well plate. The optical density (540 nm) of each sample was read starting one hour after the first addition of ampicillin and directly preceding each addition thereafter.

### 6.2.6 Activity spectrum screening

The activity of *L. lactis* L57 against the *S. iniae* collection was determined by an altered deferred antagonism method (Melancon and Grenier, 2003). An overnight *L. lactis* 57 culture was streaked in a single line on an HIA plate with a sterile loop. Isolates from

the study collection (Table 3.1) and a control of uninoculated HIB were then streaked in parallel at right angles to the L57 producing streak with sterile loops. Zones of inhibition were measured after aerobic incubation at 28°C for 16 hours. Streak plates were also made to determine the activity of *L. lactis* 57 against resistant human pathogens including *E. coli* B597, *E. coli* 53e, community acquired methicillin-resistant *Staphylococcus aureus* and *Streptococcus pneumoniae*. One plate was incubated at 37°C with $CO_2$ and the other was incubated aerobically at 37°C to ensure proper growth of the pathogens. Zones of inhibition were measured after 24 hours.

### 6.2.7 Plasmid curing of *L. lactis* L57

As a preliminary test of the possibility that the genes encoding BLIS-L57 are plasmid encoded, plasmid curing of *L. lactis* L57 was attempted. Colistin sulphate and oxolinic acid agar (CSOA; Appendix 1.4) is a Gram-positive selective agar that contains oxolinic acid (Nguyen and Kanai, 1999) and was therefore used in an attempt to cure *L. lactis* L57 of plasmid encoded genes. *L. lactis* L57 was streaked onto a CSOA plate and grown aerobically at 28°C overnight. A pure colony was removed from the plate, inoculated in HIB, and incubated aerobically for 18 hours. This culture was then prepared as in section 6.2.2 to produce a CSOA treated cfs. The treated cfs was assayed for activity using the spot-on-lawn assay and the microtitre plate assay.

### 6.2.8 Effect of heat, pH and enzymes on activity

To determine the effect of heat on BLIS-L57 activity, cfs samples were heated at 100°C for 10, 20, 30 and 60 minutes. Following removal from 100°C, the cfs samples were allowed to cool to room temperature before being used in activity assays. To determine the effect of pH on BLIS-L57 activity, cfs samples were adjusted to pH levels between 1.5 and 9.5 using 1N NaOH or HCl. Samples were incubated aerobically at 28°C for 2 hours, then readjusted to pH 5 (the pH level of untreated cfs following incubation) before being used in activity assays. After heat and pH treatment, the remaining antagonistic activity was assayed using the spot-on-lawn and microtitre plate assays.

To determine the effect of different enzymes on BLIS-L57 activity, cfs samples were adjusted to pH 7.0 and then treated to a final concentration of 2 mg $ml^{-1}$ with the following enzymes: proteinase K (Sigma; 40 units $mg^{-1}$), α-chymotrypsin (Sigma; 59.3 units $mg^{-1}$), trypsin (Sigma; 2.6 units $mg^{-1}$), pepsin (Sigma), papain (Sigma; 19 units $mg^{-1}$), and catalase (Sigma; 1340 units $mg^{-1}$). Samples were incubated at 37°C with shaking for 2 hours, then at 100°C for 5 minutes to deactivate enzymes. A cfs untreated with enzymes was used as a control. Remaining antagonistic activity was assayed as present or absent using the spot-on-lawn assay.

### 6.2.9 Kinetics of production

The kinetics of BLIS-L57 production by *L. lactis* L57 was investigated by measuring the growth of the bacterium over time as well as the activity of BLIS-L57 produced over the same period. Three milliliters overnight growth of *L. lactis* L57 was added to 80 ml HIB (3.75% vol/vol) and the culture was incubated aerobically at 28°C with shaking. The optical density of the culture was measured at 600 nm every hour until 12 hours growth, then periodically up to 28 hours growth. At each optical density reading, 1 ml culture was removed and used to create a cfs sample, which was stored at 4° until used. Following the collection of all cfs samples, antagonistic activity was measured using a spot-on-lawn assay.

### 6.2.10 Activity

The activity of BLIS-L57 was determined as either bacteriostatic or bactericidal by measuring the growth of indicator strain *S. iniae* S23 after the addition of BLIS-L57. Early log-phase growth of *S. iniae* S23 was distributed into three 10 ml aliquots. One milliliter cfs was added to two of the S23 cultures. One milliliter filtered HIB was added to the other S23 culture as a control. The optical density of the three cultures was measured at 600 nm periodically over 24 hours. In another experiment to measure cell viability of the treated culture, mid log-phase growth of *S. iniae* S23 was distributed into two 10 ml aliquots. One milliliter of cfs was added to one aliquot and 1 ml filtered HIB was added to the other as a control. At time intervals following the addition of the cfs,

optical density measurements at 600nm were taken and 10-fold serial dilutions were grown on HIA to measure cell forming units (cfu) of the indicator in both cultures. Spread plates were checked for cfu at 48 hours.

### 6.2.11 Protein purification

*L. lactis* L57 was grown overnight in HIB that had been previously filtered through Millipore type HA filters (to remove unwanted proteins from the media). Cell-free supernatant was produced as described previously in section 6.2.2. A portion of the cfs was treated with ammonium sulphate and another portion was filtered through a Millipore type HA filter.

#### 6.2.11.1 Ammonium sulphate precipitation

The ammonium sulphate precipitation was performed in two stages. For the primary precipitation, saturated ammonium sulphate solution (Appendix 1.8) was added slowly to 100 ml cfs while stirring to 60% saturation at room temperature. The solution was agitated 12 hours at 4°C, then centrifuged at 4500 *g* for 30 minutes at 4°C. The precipitate was resuspended in 10 ml sterile distilled water. A small portion of this primary precipitate sample was removed and stored at 4°C for the activity assay. Saturated ammonium sulphate solution was added slowly to the remaining primary solution while stirring to 80% saturation at room temperature. This solution was agitated 12 hours at 4°C, then centrifuged at 4500 *g* for 30 minutes at 4°C. The secondary precipitate was resuspended in 1 ml (1% original volume) sterile distilled water and stored at 4°C until used.

#### 6.2.11.2 HA filtration

A 100 ml portion of untreated cfs was filtered through a Millipore type HA filter with high protein affinity. The filtrate was stored at 4°C for the activity assay. The HA filter was dissolved in 3 ml acetone and the acetone was evaporated under vacuum at 33°C for

3h. The proteins were resuspended in 400 µl (0.4% original volume) sterile distilled water and stored at 4°C until used.

### 6.2.12 Activity assay of partially purified proteins

The activity of the HA derived and ammonium sulphate precipitated (primary and secondary) proteins was tested with a microtitre plate assay along with the filtrate from the HA filtration to confirm the retention of the inhibitory substance by the filter.

### 6.2.13 Electrophoresis of partially purified proteins

Approximate molecular size of BLIS-L57 was determined by tris-glycine and tris-tricine SDS-PAGE. Aliquots of the partially purified protein samples and an aliquot of untreated cfs were mixed 1:1 with TruSep SDS sample buffer (NuSep, Australia), boiled for 5 min, and centrifuged at 3300 *g* for 5 min according to the manufacturer's instructions. Samples were run on 4-20% gradient tris-glycine gels (NuSep, Australia) with a prestained protein ladder (Fermentas, Australia) at 40mA constant using a Mini Protean electrophoresis system (BioRad, Australia).

The protein samples were also prepared with TruSep Tricine SDS sample buffer (NuSep, Australia) and run on 16% Tris-tricine gels (NuSep, Australia) with a low MW prestained protein ladder (Fermentas, Australia) or PageRuler low range unstained protein ladder (Fermentas, Australia) at 150V constant. Gels were either stained overnight with Gradipure after rinsing in distilled water, then destained with 10% acetic acid solution (Appendix 1.9), or silver stained using a Silver Stain Plus staining kit (BioRad, Australia) according to the manufacturer's protocol.

Proteins separated by SDS-PAGE were eluted from the gel using a modified protocol from Busarcevic *et al.* (2008). Protein bands were excised from the gel, sliced into small pieces and covered with protein elution buffer (Appendix 1.10). Following agitation at room temperature overnight, the elution samples were centrifuged at 10,000 *g* for 5 min and the supernatants concentrated by vacuum at 33°C for 20 min. Two controls were used: one elution made with a slice of gel from an unused lane

(containing no protein) and one with elution buffer only. Each eluted sample was assayed for antimicrobial activity with a spot-on-lawn assay along with the controls.

## 6.3 Results

### 6.3.1 Identification of BLIS producer

The API 20 Strep test returned the result of *Lactococcus lactis* ssp. *lactis* with low discrimination at 75.4%. The API rapid ID 32 Strep test returned a doubtful result of *Lactococcus lactis* at 98.8%. The strain did not group within Lancefield groups A-G, and did not grow at 45°C or in 6.5% NaCl. The PCR assay for the lactate oxidase (*lctO*) gene of *S. iniae* using the primer combination LOX-1/LOX-2 (Mata *et al.*, 2004a) returned a negative result for strain L57. The same primer combination PCR run on an isolate of *Lactococcus lactis* ssp. *lactis* was negative (Mata *et al.*, 2004a). The 16S PCR assay using universal primers 27F/1492R returned a sequence sharing 99% identity with *Lactococcus lactis* ssp. *lactis* in BLAST (basic local alignment search tool; National Library of Medicine, United States). According to sequencing and antagonism tests with *L. lactis* isolate L49 (data not shown), *L. lactis* L57 is the same strain as L49, originally isolated from a moribund sleepy cod (*Oxyeleotris lineolatus*) at the School of Veterinary and Biomedical Sciences, JCU.

### 6.3.2 Confirmation of bacteriocin-like inhibitory activity

*L. lactis* L57 grew on the lawn of *S. iniae* 56, surrounded by a zone of inhibition (Figure 6.1). The cfs produced only a clear plaque on the lawn. The unfiltered L57 spots prevented growth of *S. iniae* S56 through the solid media flip plate (Figure 6.1B). One cfs spot also produced clearance through the media, but the zone of inhibition was not as large.

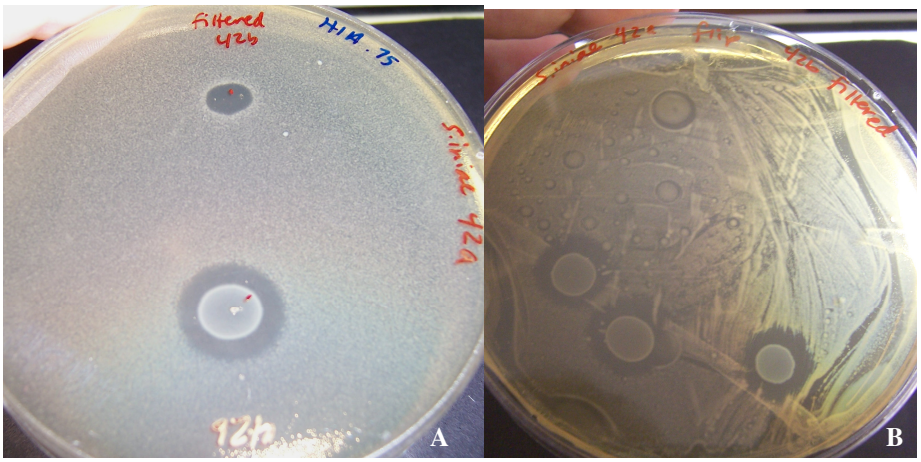


**Figure 6.1** Preliminary antagonistic tests with *L. lactis* L57. A: L57 (bottom) and cfs (top) spotted directly on *S. iniae* S56 lawn. B: L57 (bottom) and cfs (top) spotted on flip plate of S56 lawn.

The indicator lawn spotted with *L. lactis* L57 that was grown in anaerobic conditions revealed larger clearance zones than the lawn grown aerobically. The anaerobic lawn showed clearance zones of 2 mm around the growth of *L. lactis* L57, but the aerobic lawn showed clearance zones of only 1.5 mm (Figure 6.2). The aerobic lawn displayed a weaker, more diffuse zone of clearance outside of the strong clearance, which spread for another 1-1.5 mm.

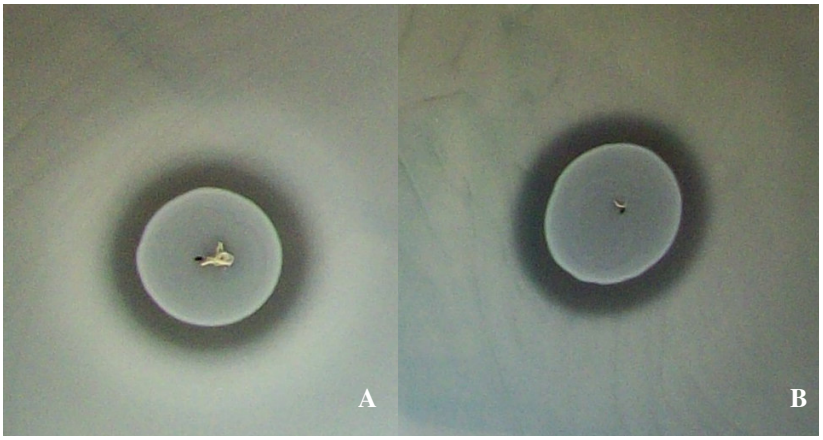


**Figure 6.2** *L. lactis* L57 spotted onto indicator lawns of *S. iniae* S23 and grown aerobically (A) and anaerobically (B).

### 6.3.3 Activity Spectrum of *L. lactis* L57

*L. lactis* L57 limited the growth of all bacterial isolates in the deferred antagonism assay except *S. iniae* isolates S32, S39, S47, *L. lactis* L49, *Enterococcus faecalis* E50, and *Citrobacter freundii* isolates C54 and C55 (Figures 6.3 and 6.4). *L. lactis* L57 therefore produced antagonistic activity against 93.75% (45/48) of *S. iniae* isolates in the JCU library. *L. lactis* L57 failed to inhibit the growth of the tested human pathogens.

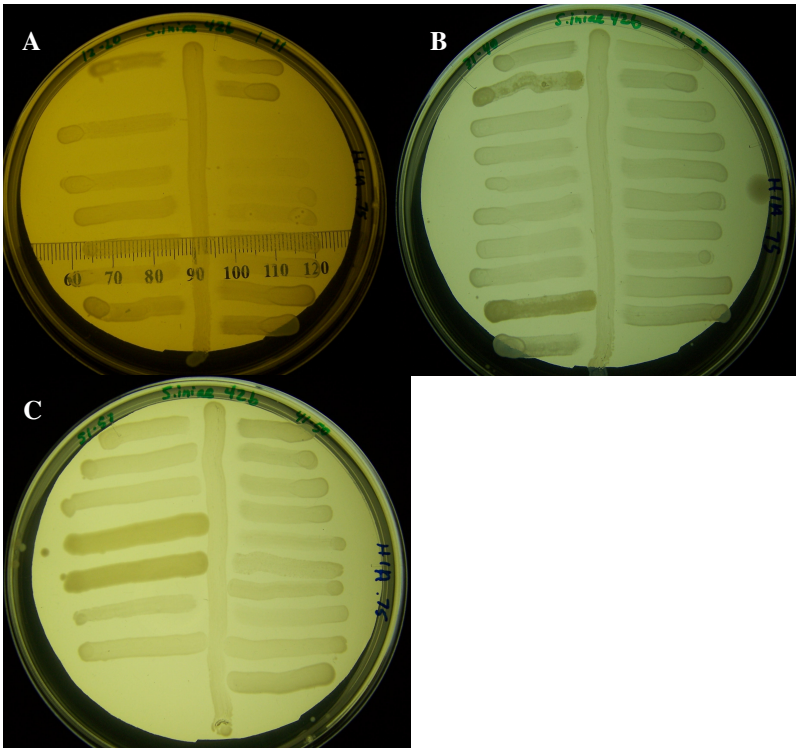


**Figure 6.3** Deferred antagonism assay showing all bacterial isolates from the collection streaked away from *L. lactis* L57. A: Strains 1-20 with *L. lactis* L57 (1-11 on right, 12-20 on left). B: Strains 21-40 with *L. lactis* L57 (21-30 on right, 31-40 on left). C: Strains 41-57 with *L. lactis* L57 (41-50 on right, 51-57 on left).

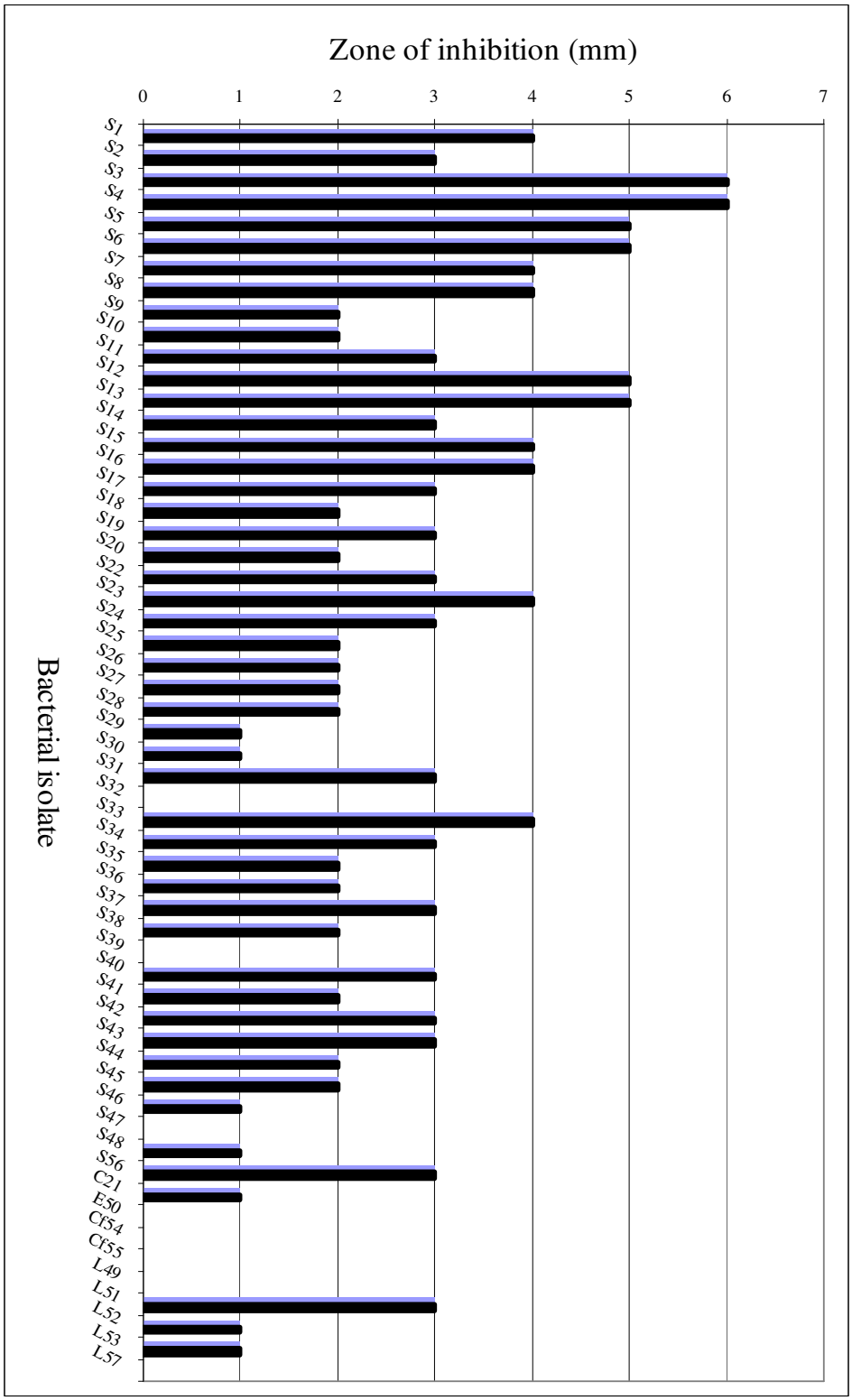


**Figure 6.4** Zones of inhibition of bacterial strains produced by *L. lactis* 57 in the deferred antagonism assay. Isolate prefixes: (S) *Streptococcus iniae*, (C) *Carnobacterium divergens*, (E) *Enterococcus faecalis*, (Cf) *Citrobacter freundii*, (L) *Lactococcus lactis* ssp. *lactis*.

### 6.3.4 Ampicillin test

It was difficult to measure a decrease in bacterial growth by optical density measurements unless the inhibitor was added within 1.5 hours of the beginning of the experiment, which corresponded to the indicator strain having an $OD_{540}$ of 0.52 or less (Figure 6.5). This experiment resulted in the inhibiting substance being added to an indicator with an optical density of less than $OD_{540}$ 0.5 for all further experiments in order to measure a substantial decrease in bacterial growth during liquid assays of growth inhibition.

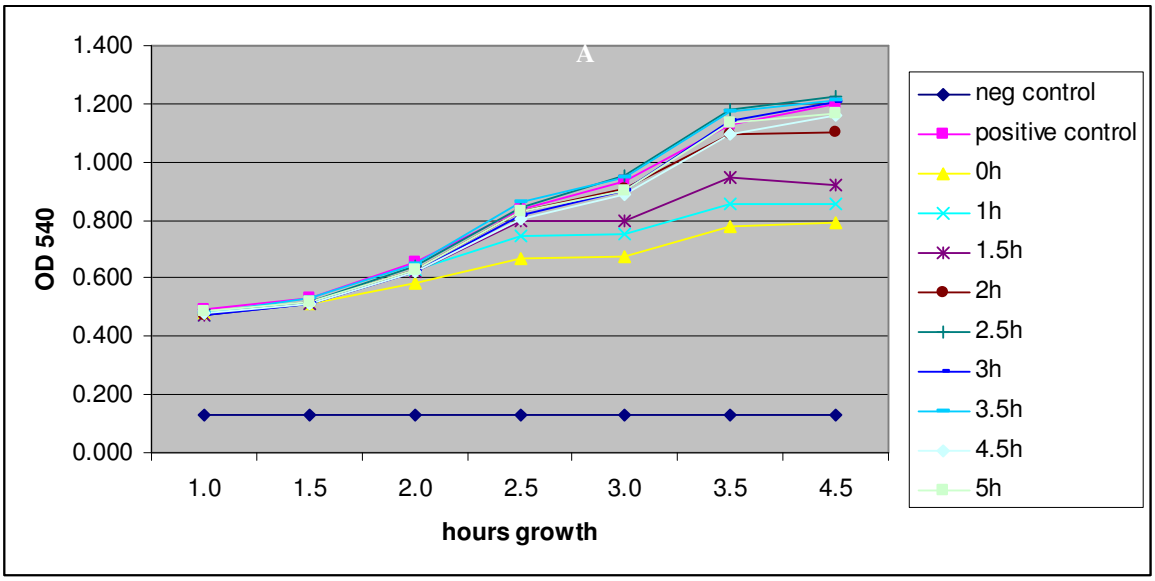


**Figure 6.5** Optical density measurements of *S. iniae* S23 after additions of ampicillin at the indicated times.

### 6.3.5 Plasmid curing of *L. lactis* L57

The CSOA treated cfs had similar activity to the untreated cfs (Table 6.1 and Figure 6.6).

### 6.3.6 Effect of heat, pH and enzymes on activity

Table 1 displays the properties of BLIS-L57 cfs. The cfs activity remained following 60 minutes at 100°C and showed activity over a broad pH range (pH 2.5-9.5), though activity was weaker at pH levels higher than 5.5. The cfs also showed antagonistic activity against the indicator after exposure to pepsin and catalase, but lost activity when exposed to proteinase K, α-chymotrypsin, trypsin and papain.

**Table 6.1** Antagonistic activity by BLIS-L57 from *L. lactis* L57 against indicator *S. iniae* S23 following different treatments. Activity was measured as present (+) or absent (-) for the enzyme tests. Absence of antagonistic activity (-) correlates to senstivity (s) of BLIS-L57 to the enzyme tested, and antagonism of the indicator (+) correlates to resistance (r) of BLIS-L57 to the enzyme tested.

| **Treatment** | **Activity (AU ml$^{-1}$)** |
|---|---|
| Control cfs | 160 |
| CSOA | 160 |
| *Heat* | |
| 10m @100°C | 80 |
| 20m @100°C | 80 |
| 30m @100°C | 80 |
| 60m @100°C | 80 |
| *pH* | |
| 2.5 | 160 |
| 3.5 | 160 |
| 5.5 | 160 |
| 6.5 | 80 |
| 7.5 | 80 |
| 8.5 | 80 |
| 9.5 | 80 |
| *Enzymes* | |
| Proteinase K | - (s) |
| α-chymotrypsin | - (s) |
| Trypsin | - (s) |
| Pepsin | + (r) |
| Papain | - (s) |
| Catalase | + (r) |
| *Ammonium sulphate* | |
| 1° precipitation | 1280 |
| 2° precipitation | 10240 |
| *HA filtration* | |
| retained activity | 240 |
| filtered activity | 80 |

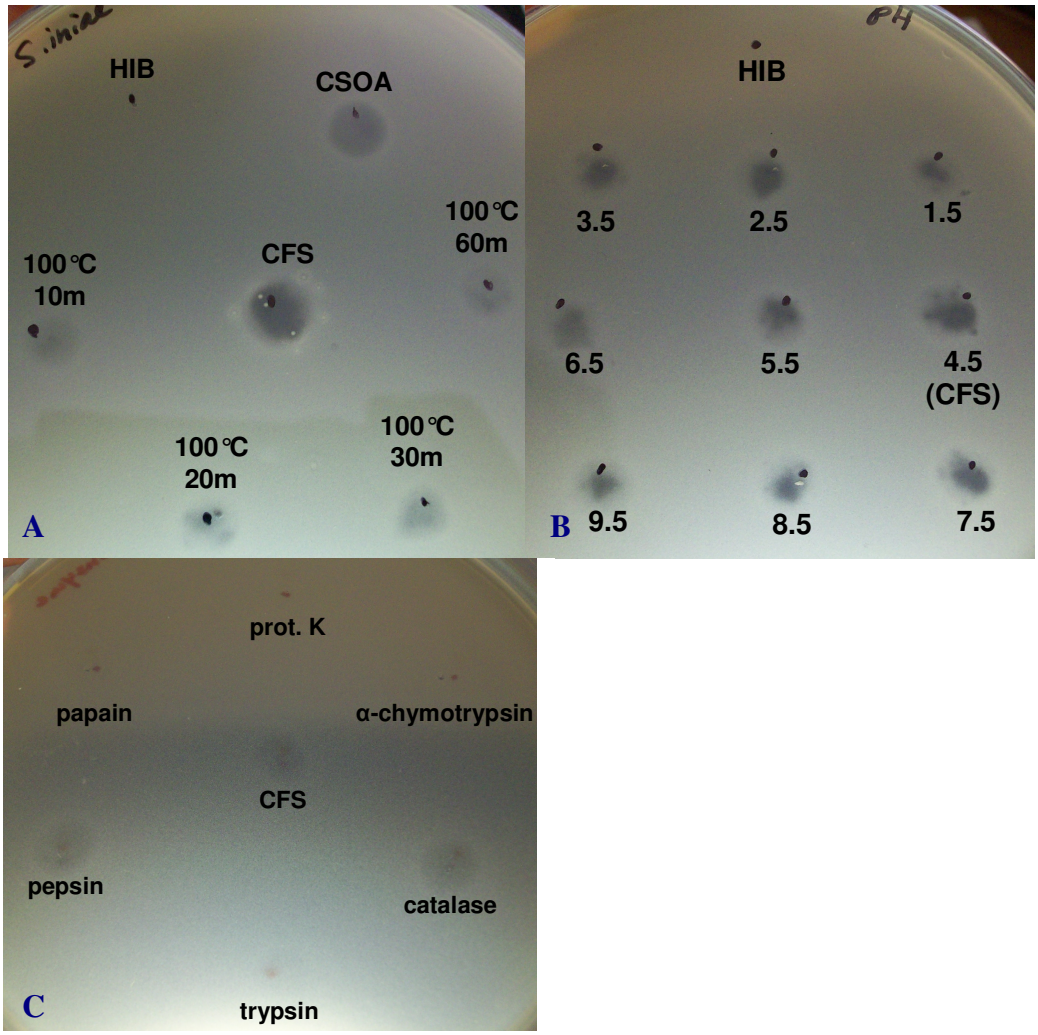


**Figure 6.6** Spot-on-lawn assays for heat (A), pH (B), and enzyme (C) effects on the activity of BLIS-L57. Soft top lawns seeded with *S. iniae* S23 were used as indicators.

### 6.3.7 Kinetics of production

The kinetics of BLIS-L57 production by *L. lactis* L57 grown in HIB are shown in Figure 6.7. *L. lactis* L57 began producing BLIS-L57 after 4 hours growth, which corresponded to the end of the log growth phase. BLIS-L57 production reached a maximum at early stationary phase, and then began to drop following 11 hours growth. The activity is apparently unstable as the measurements of BLIS-L57 clearance continued to decrease throughout 24 hours.

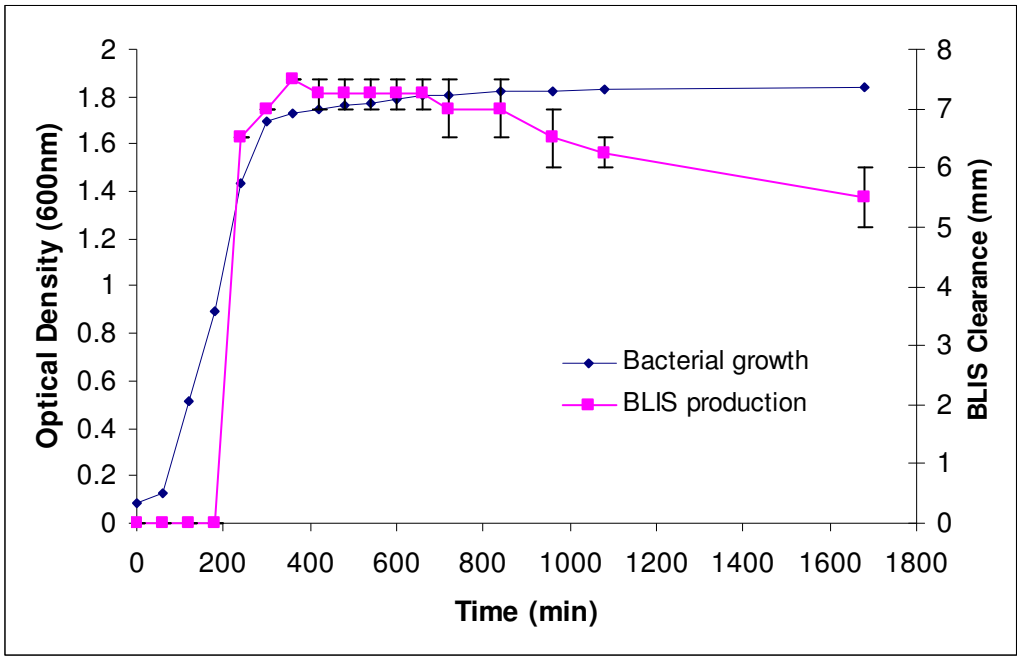


**Figure 6.7** Kinetics of BLIS-L57 production during the growth of *L. lactis* 57. The growth curve of the bacteria was generated by measurements of $OD_{600}$ and BLIS-L57 production was evaluated by measuring the clearance produced on indicator *S. iniae* S23 by cfs samples collected throughout the period shown.

### 6.3.8 Activity

The activity of BLIS-L57 against a bacterial indicator is shown in Figures 6.8 and 6.9. The growth of the indicator strain S23 not only stopped following the addition of BLIS-L57, but the optical density continued to drop steadily over time without recovery, while the control culture grew normally to an optical density of 1.613 measured at 600 nm (Figure 6.8). The cell viability of the treated indicator culture dropped by at

least 10-fold in the first 30 minutes following the addition of BLIS-L57 and continued to drop over 70 minutes while the control culture cfu increased 10-fold (Figure 6.9).

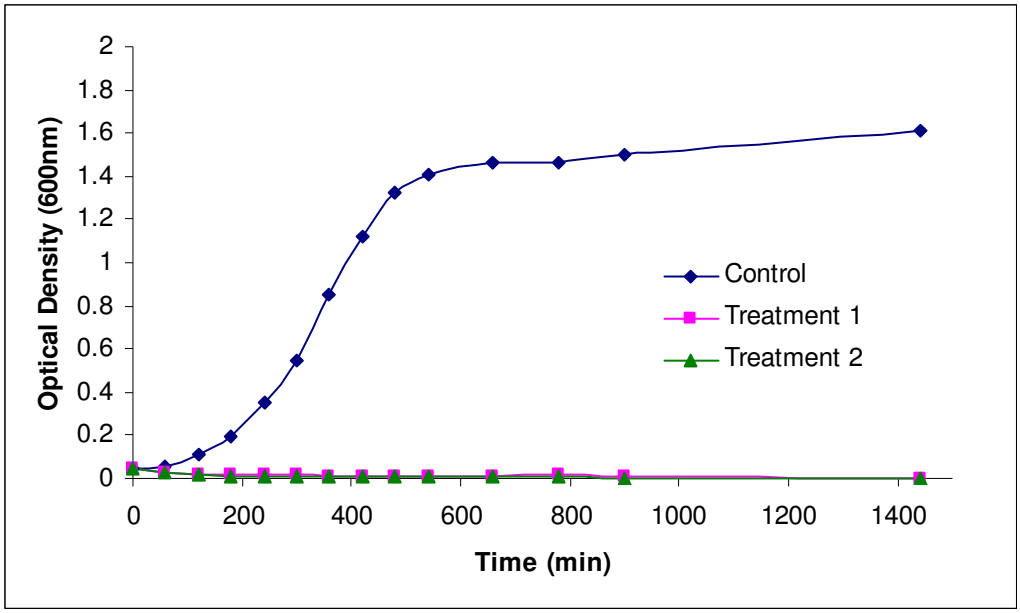


**Figure 6.8** Mode of action of BLIS-L57 on the growth of *S. iniae* S23. The BLIS-L57 cfs (treatment cultures) or the HIB supernatant (control) was added directly after the zero minute reading (during early exponential growth phase).

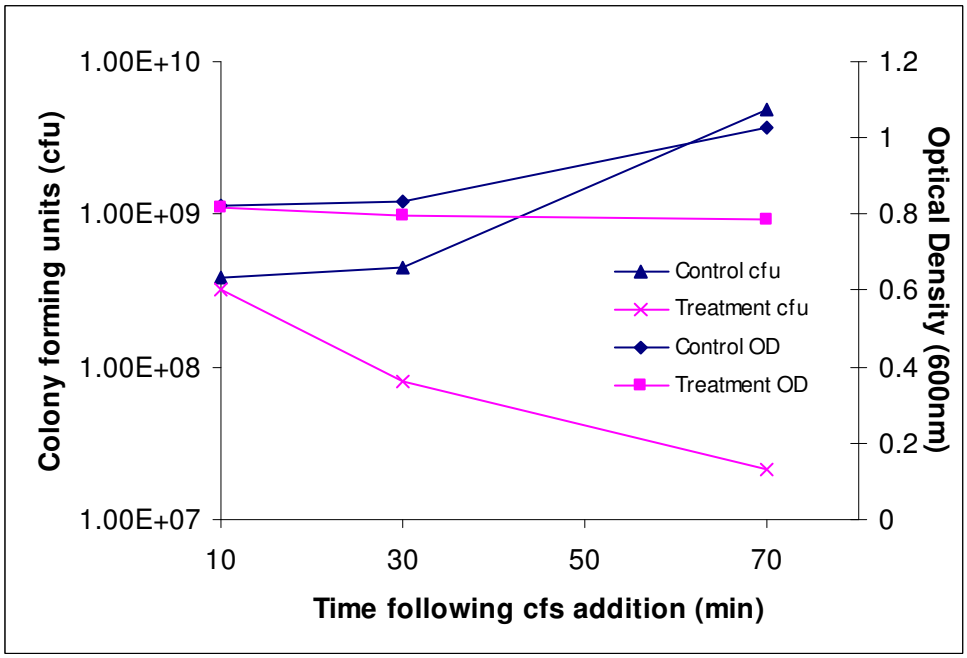


**Figure 6.9** Effects of BLIS-L57 on the cell viability of *S. iniae* S23. The BLIS-L57 cfs (treatment culture) or the HIB supernatant (control) was added during mid log-growth phase.

### 6.3.9 Protein concentration

The ammonium sulphate precipitation was highly successful at concentrating the bacteriocin (Table 6.1). The primary precipitate showed 8 times the activity (in AU) of the untreated cfs and the secondary precipitate showed at least 64 times the activity of the untreated cfs, as 1/512 was the highest dilution made of the substances tested (in 50 µl volumes) with the microtitre assay. HA filtration also concentrated the bacteriocin, but the HA derived proteins produced less than 2 times the antagonistic activity of the untreated cfs on average. The filter did not retain all of the bacteriocin protein; the filtrate also produced antagonistic activity on the indicator.

### 6.3.10 Protein electrophoresis

Following tris-glycine SDS-PAGE of the concentrated and untreated cfs, the secondary ammonium sulphate precipitated proteins produced the strongest bands on the gel, with bands present at approximately 54 kDa, between 24 and 35 kDa and at approximately 17kDa (Figure 6.10). The low molecular weight proteins did not separate well on tris-glycine gel and resulted in a rough smear. This low molecular weight smear was also produced by the HA-derived protein sample. The cfs and HA filtrate samples were not concentrated enough to produce visible protein bands on the gel.

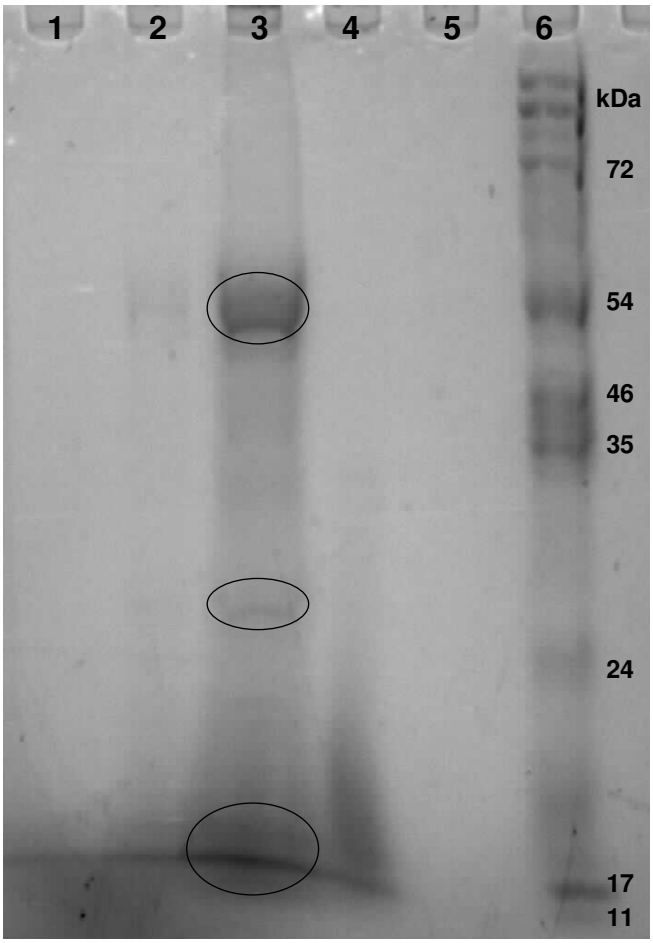

**Figure 6.10** Tris-glycine SDS gel showing protein separations from different concentrations of BLIS-L57 produced by *L. lactis* L57. Lane 1: untreated cfs; lane 2: primary ammonium sulphate precipitate; lane 3: secondary ammonium sulphate precipitate; lane 4: HA derived proteins; lane 5: HA filtrate; lane 6: protein standards.

The secondary ammonium sulphate precipitated protein sample was run in triplicate on tris-glycine to obtain the bands used for protein elution (Figure 6.11A). Following elution of proteins from the tris-glycine gel slices, antagonistic activity was produced on the indicator by the small sized protein smear (observed around 17 kDa) and also to a lesser extent by the large molecular weight protein band (54 kDa) (Figure 6.11B). No antagonism was produced by the middle protein band between 24 and 35 kDa in size.

The tris-tricine gel allowed greater separation of the low molecular weight proteins, and the small protein band from all samples was clarified at approximately 5 kDa (Figures 6.12 and 6.13). Silver staining allowed greater clarification of proteins; two separate protein bands became visible around 20 kDa (Figure 6.12). Following elution of

proteins from the gel slices, only minor antagonistic activity was produced on the indicator by the small molecular weight protein band (5 kDa) but strong antagonism was produced by the large molecular weight (54kDa) protein band (Figure 6.13B).

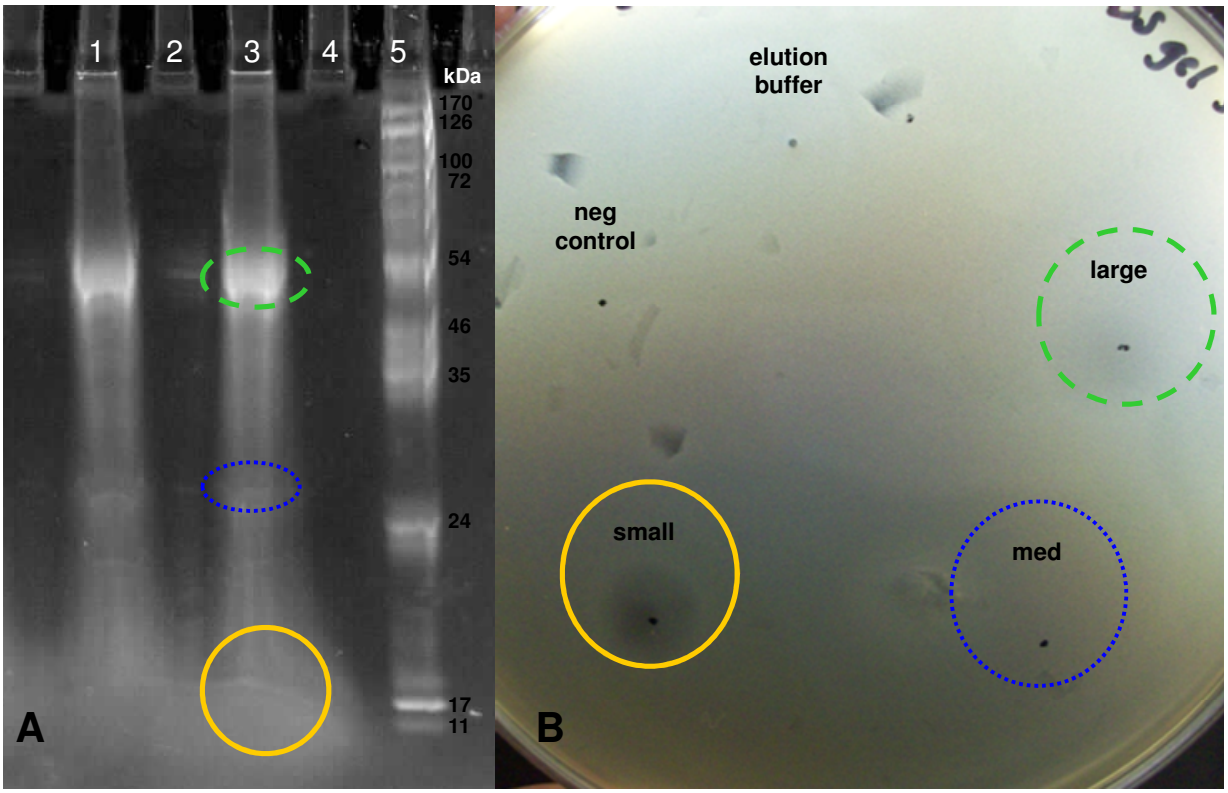


**Figure 6.11** A: 4-20% gradient tris-glycine SDS-PAGE gel showing triplicate protein separations of ammonium sulphate concentrated BLIS-L57 from *L. lactis* L57. Lanes 1, 3: secondary ammonium sulphate precipitated proteins; lane 5: protein standards. B: Spot-on-lawn antagonism assay on *S. iniae* S23 for proteins eluted from tris-glycine SDS gel run of concentrated BLIS-L57 from *L. lactis* L57.

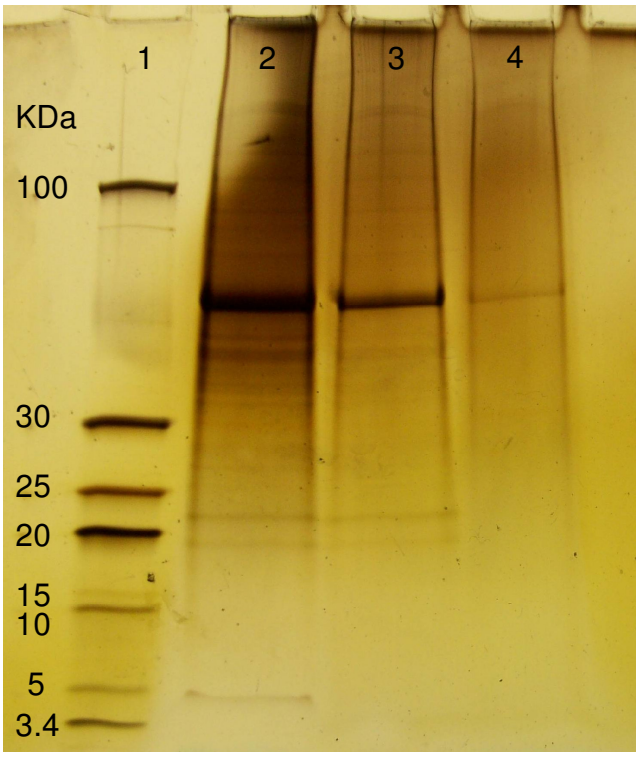


**Figure 6.12** Silver stained 16% tris-tricine SDS-PAGE showing protein separations of ammonium sulphate and HA concentrated BLIS-L57 from *L. lactis* L57. Lane 1: protein standards; lane 2: secondary ammonium sulphate precipitate; lane 3: primary ammonium sulphate precipitate; lane 4: HA derived proteins.

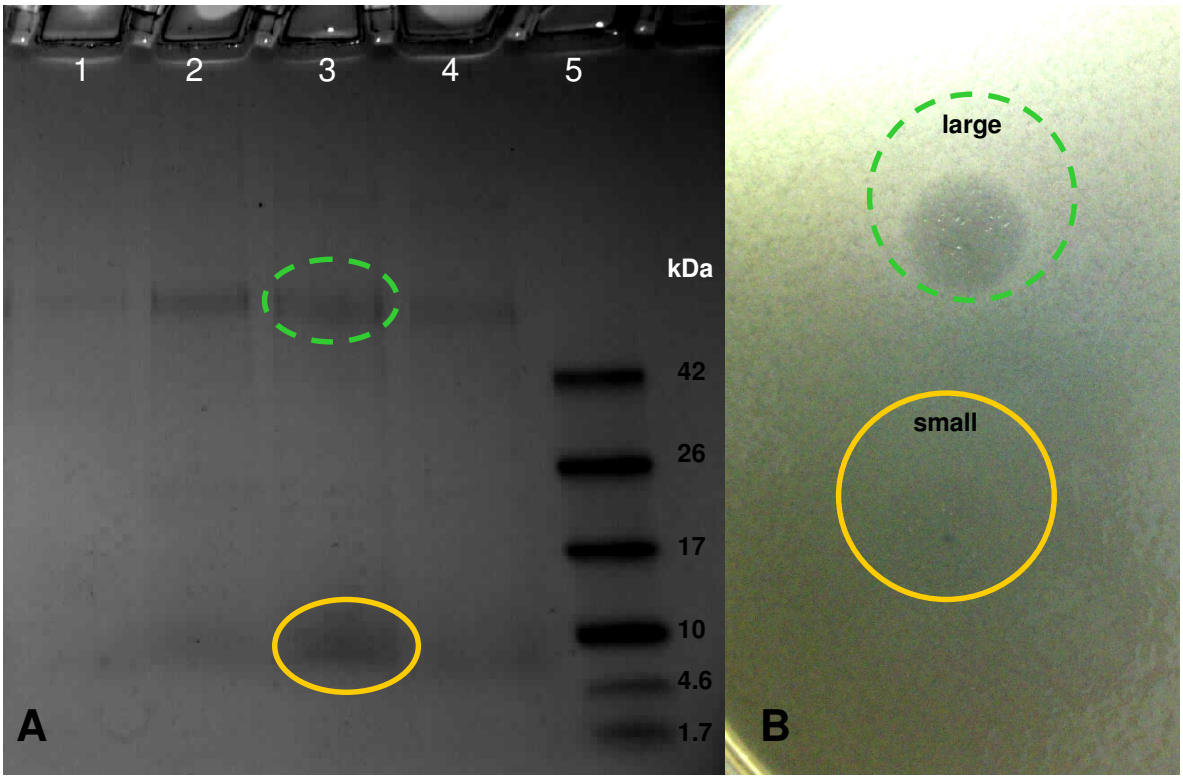


**Figure 6.13** A: 16% tris-tricine SDS gel showing protein separations of ammonium sulphate and HA concentrated BLIS-L57 from *L. lactis* L57. Lane 1: HA derived proteins; lane 2: primary ammonium sulphate precipitate; lanes 3, 4: secondary ammonium sulphate precipitate; lane 5: protein standards. B: Spot-on-lawn antagonism assay on *S. iniae* S23 for proteins eluted from tris-tricine SDS gel run of concentrated BLIS-L57 from *L. lactis* L57.

## 6.4 Discussion

An inhibitory substance is recognized as a bacteriocin if it meets the criteria of being a biologically active protein moiety with a bactericidal mode of action (Tagg *et al.*, 1976). According to this definition, BLIS-L57 produced by *L. lactis* L57 has been identified as a bacteriocin (now designated as BacL49, due the identification of its original producing strain). The substance BacL49 is produced in anaerobic conditions and is capable of diffusing through solid media, so hydrogen peroxide and phage activity were ruled out as the cause of antagonism according to Tagg *et al.* (1976). The sensitivity of BacL49 to a number of serine and cysteine proteases confirmed it is protein in nature. Finally, mode of action was confirmed as bactericidal by optical density and cell viability experiments.

BacL49 has a broad activity spectrum against *S. iniae*, showing antagonism against 93.75% (45/48) of isolates in the JCU library. This is currently the only known report of *S. iniae* sensitivity to a lactococcal bacteriocin. The bacteriocin producer studied, *L. lactis* L57, is the same strain as L49, originally isolated from a freshwater fish as stated previously (Section 6.3.1). Strain L49 was originally misidentified as *S. iniae* upon isolation, but was not collected during an *S. iniae* outbreak. Other bacteriocin-producing *L. lactis* strains have been isolated from freshwater fish in the past (Campos *et al.*, 2006; de Kwaadsteniet *et al.*, 2008), however these studies tested bacteriocin activity against *S. aureus*, *L. monocytogenes*, and other pathogens important in food spoilage and human infection. This is the first known study to discuss and test a lactoccocal bacteriocin as a treatment of a fish pathogen.

Bacteriocins are generally produced concomitantly with immunity proteins that provide cross-immunity for bacteria producing related bacteriocins (Jack *et al.*, 1995), and thus the producing strain *L. lactis* L57 (as well as L49) were insensitive to BacL49. It is possible that BacL49 may show a broad activity spectrum across other Gram-positive bacteria, demonstrated by the susceptibility of some other *Lactococcus* strains tested, but a wide range of Gram-positive bacteria will need to be tested to confirm this hypothesis.

Bacteriocins are often associated with plasmids or transposons (Broda, 1979). Oxolinic acid, contained in CSOA selective agar, is one of the 4-quinolone antimicrobial agents, which have been shown to result in plasmid elimination when used in treatment of carrier bacteria (Crumplin and Smith, 1981). However, growth of *L. lactis* L57 on CSOA failed to cure the strain of bacteriocin production, suggesting that BacL49 is not associated with a plasmid. Most lantibiotic gene clusters are plasmid regulated (Riley and Wertz, 2002), so other curing methods should be trialled to confirm that BacL49 is in fact encoded on the bacterial chromosome. A plasmid purification from *L. lactis* L57 could be performed, followed by transfer of the plasmid(s) by conjugation to plasmid-free strains of *L. lactis*. If plasmid curing resulted in bacteriocin-negative derivatives of *L. Lactis* L57, and plasmid transfer resulted in bacteriocin production by transconjugated strains, the bacteriocin would be shown to be plasmid encoded (Neve *et al.*, 1984).

BacL49 is heat stable, maintaining activity levels of 80 AU $ml^{-1}$ after 60 minutes at 100°C, and is also stable over a pH range of 2.5-9.5, though antagonistic activity was twice as high when the bacteriocin was incubated in an acidic environment (pH 2.5-5). Most bacteriocins are more tolerant of acidic rather than alkaline pH levels (Tagg *et al.*, 1976; Campos *et al.*, 2006). An acidic pH can be necessary for the retention of the cationic properties of bacteriocin peptides, which appear to be crucial for their antagonistic activity (Mortvedt-Abildgaard *et al.*, 1995). BacL49 sensitivity to serine proteases proteinase K, α-chymotrypsin and trypsin and the cysteine protease papain confirms it is a protein moiety as stated previously. Most *L. lactis* bacteriocins are heat stable and tolerant to acidic conditions (Fang and Yang, 1995; Rodríguez *et al.*, 1995; Campos *et al.*, 2006; de Kwaadsteniet *et al.*, 2008), but many show different enzyme sensitivities. Nisin F, also produced from an *L. lactis* isolated from fish, shows resistance to pepsin similar to BacL49, but was resistant to Proteinase K (de Kwaadsteniet *et al.*, 2008). No published descriptions of *L. lactis* bacteriocins matched the exact protease sensitivity profile of BacL49.

Production of BacL49 begins at the end of log growth phase, but activity levels decline throughout stationary phase. Production of Gram-positive bacteriocins primarily occurs during late log phase and early stationary phase (Riley and Wertz, 2002), and similar activity declines have been reported from some *L. lactis* bacteriocins (Fang and Yang, 1995), but certainly not from all. Campos *et al.* (2006) found the stability of bacteriocin production by another *L. lactis* isolate was dependent on the indicator strain tested. Bacteriocin production is often cell-density dependent, and quorum-sensing enables the producer to turn on production during times of increased competition for resources (Eijsink *et al.*, 2002).

The reduction in optical density and viable cell count of the BacL49 treated indicator culture over time indicates a bactericidal mode of action. Many lantibiotics kill bacteria by targeting lipid II, thus blocking cell wall synthesis and leading to cell lysis by pore formation (Hasper *et al.*, 2006). The bactericidal mode of action of BacL49 could be confirmed as bacteriolytic by TEM studies or measurement of intracellular enzyme release (Morgan *et al.*, 1995; Martinez-Cuesta *et al.*, 2000).

The production of antagonistic activity by the two differently sized peptide bands eluted from SDS gels (54 kDa, and 5 kDa) could demonstrate that two different inhibitory substances are being concurrently produced by *L. lactis* L49. The two component lantibiotic lacticin 3147 consists of two 3-4kDa peptides, but both are required for antagonistic activity (McAuliffe *et al.*, 2000), which is not the case with the BacL49 peptides as evidenced by their independent production of antagonism. It is also possible that cleaving of BacL49 is occurring, or that the large molecular weight peptide is simply an uncleaved quaternary structure of the small peptide.

Ammonium sulphate concentration was extremely effective at concentrating BacL49, especially following two rounds of precipitation. Purification by ammonium sulphate could not be assessed as untreated cfs concentrations were not high enough to produce peptide bands on SDS gels. However, an increase in antimicrobial activity by bacteriocins during purification has been previously reported (Aymerich *et al.*, 1996),

and this could indicate that the substantial increase in activity following ammonium sulphate concentration is also due to partial purification. Millipore type HA filters have previously been used to collect and purify bacteriocin proteins, with a reported 80% recovery of activity (Morgan *et al.*, 1995). The HA filters were ineffective at retaining all bacteriocin proteins in this study, displayed by the activity of the filtrate on indicator lawns. However, the filters were successful at concentrating the BacL49 peptides, evidenced by visible bands on SDS gels in contrast to untreated cfs. On the tris-glycine gradient gel, the HA protein sample only produced a small peptide band, unlike the other samples, suggesting the filter only retains the small-sized peptide and that the activity produced by the filtrate was caused by the large peptide. However, a faint band appeared for the large peptide on the tris-tricine gel, indicating that the filter must show some affinity for the large peptide as well.

This study suggests that BacL49 could potentially be used for treatment of early-stage *S. iniae* infections in fish. As with bacteriophages, the narrow killing spectrum of bacteriocins makes them good candidates for targeting specific pathogenic bacteria (Riley and Wertz, 2002), but the broad activity spectrum of BacL49 on different isolates of *S. iniae* would prove to be an advantage due to the large variation in strains of this pathogen (Kvitt and Colorni, 2004; Eyngor *et al.*, 2008). The fact that BacL49 remains active over a broad pH range is also advantageous due to the fact that *S. iniae* can establish in a variety of organs and tissues depending on the species of fish infected. The heat tolerance of BacL49 could allow the bacteriocin to be delivered to fish in a processed food form.

Thorough purification and elucidation of the amino acid make-up of BacL49 should be the next steps undertaken before *in vivo* testing in order to minimize immunological reaction of the fish to the treatment. Pathological effects of bacteriocins must be considered before they are used in an *in vivo* situation, and it would be beneficial to determine whether the bacteriocin is strongly antigenic (Tagg *et al.*, 1976). However, most bacteriocins are not toxic to animals at effective antimicrobial concentration due to their specificity (Kobayashi *et al.*, 2001).

Spontaneous resistance of bacterial cells to lantibiotics has been observed, though a high multiplicity of infection was necessary to induce a significant frequency of resistance (Ming and Daeschel, 1993). The cell membrane mutations necessary for developed resistance to pore forming lantibiotics could reduce pathogenicity in the bacteria, similar to bacteria with developed phage resistance (Smith *et al.*, 1987; Park *et al.*, 2000). Identification of other bacteriocins with antagonistic activity against *S. iniae* would prove useful, as cocktails of different bacteriocins may reduce the formation of resistant bacteria (Vignolo *et al.*, 2000).

If bacteriocin delivery proved difficult, or adequate concentrations were hard to determine, *L. lactis* ssp. *lactis* L57 (aka L49) could be trialled as a probiotic in fish, assuming this isolate does not act as a pathogen to the host. *L. lactis* has not been documented as a fish pathogen, but is regularly present in fish and the aquatic environment (Michel *et al.*, 2007). The elucidation of the *L. lactis* genome and the fact that products from the bacteria are generally regarded as safe make the bacteria a unique candidate for genetically engineered live vaccines as well (Lan *et al.*, 2006). Alteration directly to BacL49 could also be performed to improve antagonistic activity or delivery to the pathogen. Nisin derivatives can be produced by peptide engineering to enhance antimicrobial activity against specific pathogens (Field *et al.*, 2008), and this technology could potentially be useful with BacL49.

### 6.5 Conclusion

BacL49 is a heat and pH stable bacteriocin produced by *L. lactis* ssp. *lactis* isolated from freshwater sleepy cod. BacL49 could be a nisin variant or other related lantibiotic, but elucidation of the amino acid make-up and PCR assays with nisin gene primers need to be completed. This bacteriocin is significant because it displays a broad activity spectrum for *S. iniae* isolates, implicating it as a potential new therapeutic agent for infections caused by *S. iniae* in fish. Purified BacL49 should be tested *in vivo* to determine antigenicity of the substance in fish and the bactericidal action of the

bacteriocin should be studied in depth to identify problems that may arise with bacterial resistance.

# CHAPTER SEVEN

# GENERAL DISCUSSION

Phage therapy remains a potentially effective tool for control of *S. iniae* in aquaculture, despite the inability of this study to isolate a lytic phage against the bacterium. Finding lytic phages for *S. iniae* was expected to be relatively simple due to recent successes at isolating lytic phages against other aquatic pathogens, especially with some of this successful research being produced from the same laboratories that this study was undertaken in. Due to the success of similar isolation methods used for control phages and lysogenic phages in this project, damage to virion particles was not likely the cause of failure in isolating lytic phages from environmental samples. It is more plausible that phages were not found due to a lack of proper enrichment strains or because phages were simply not collected in the environmental samples. In the future, lytic phages will likely be found on farms experiencing current outbreaks of *S. iniae*, none of which were presented during this study, probably due to the almost universal practice of vaccination against this pathogen in northern Queensland.

In hindsight, it would have been prudent to complete the lysogeny study before beginning collection and enrichment of environmental samples in order to properly exclude lysogens from the enrichments. Also, a significant amount of time was spent searching for lytic phages on spotted lawns whilst this researcher lacked experience in observing phage plaques, particularly in *Streptococcus* species. The lysogeny study and control experiments using externally sourced *S. iniae* phages added immensely to knowledge and skill level, raising some doubt as to whether lytic phage plaques were present but not observed in some early enrichment experiments.

This study found that lysogeny is present at a low rate in *S. iniae*, suggesting low superimmunity overall to whole phage therapy. However, defective prophages may be present in many strains of *S. iniae*, which could be confirmed by future sequencing

studies. The phages isolated and characterised in this study, vB_SinS-44, vB_SinS-45, vB_SinS-46 and vB_SinS-48, are the first described lysogenic phages associated with *S. iniae*. These *Siphoviridae* phages are excellent candidates for genetic modification for use in therapy, prevention or research of *S. iniae* infections in fish. These phages could be valuable for phage typing of *S.iniae* isolates and have excellent potential as tools for vaccine delivery. Sequencing of the identified *S. iniae* lysogenic phages should be undertaken as the next step, not only to identify phage genes and proteins that could be useful in therapy against the bacterium, but also to gain insight into phage-mediated virulence of this pathogen.

Considering the broad host ranges and apparently stable lytic activity of the lysogenic phages induced in this study, future research identifying phages lytic against *S. iniae* should be extremely cautious when excluding the possibility of lysogenic activity of these viruses. Repeated *in vitro* infection trials should be run to ensure the phage in question does not enter the lysogenic cycle in a range of bacterial strains. If feasible, sequencing of the phage genome could confirm the presence or absence of genes carried by lysogenic phages, such as the prophage repressor gene.

The observation that direct contact between some strains of *S. iniae* can lead to induction of prophages and consequent lysis of both bacterial strains could lead to an interesting future investigation. Could these lysogenic strains be added to an ongoing infection with a non-lysogenic strain of *S. iniae* to help reduce the levels of the original pathogen by direct antagonism? It would be interesting to discover what mechanism causes some strains and not others to induce lysis in these lysogenic bacteria. If it were an exoprotein or capsule component that could be produced and delivered without the whole bacterium, this could open up a whole new avenue of combined treatment and vaccination.

One positive outcome of the search for lytic phage was the discovery of BacL49, the bactericidal bacteriocin produced by *L. lactis* ssp. *lactis* L49. This is the first reported lactococcal bacteriocin with activity against *S. iniae*. This bacteriocin is significant

because of its heat and pH stability, and broad activity spectrum for *S. iniae* isolates, implicating it as a potential new therapeutic agent for infections caused by *S. iniae* in fish. BacL49 should be properly identified either as a form of nisin or as a unique antimicrobial peptide through PCR assays. The bacteriocin should then be purified and tested in a *S. iniae* infection model in fish to determine its efficacy *in vivo*. Delivery of BacL49 in a processed food form could easily be tested, and since BacL49 is so stable, it would likely survive the pressures and temperatures associated with pelleted fish food production, and would remain viable in the acidic environment of the fish digestive tract. BacL49 has the potential to be delivered to fish as a purified peptide or in the form of a live probiotic as *L. lactis* ssp. *lactis* L49.

Further study and manipulation of both the bacteriocin and phages isolated in this study could produce novel, effective biocontrol agents in the fight against *S. iniae*. It is likely that there is no single solution for the control of *S. iniae* in fish, but rather several solutions that when combined can greatly reduce the losses caused by this pathogen. Perhaps phages and bacteriocins could play a large role in this combined therapy and prevention regime. It may even be possible that a genetically engineered *S. iniae* lysogenic phage could insert and cause the expression of genes from BacL49, attacking the bacteria with two mechanisms at once. When several diverse agents are used to control an epizootic, the bacteria would be expected to have a severely reduced chance of evolving against all of these selective pressures at once.

The key to fighting *S. iniae* in the aquatic environment probably lies not only in the biocontrol agents used, but also in the dose of these agents. It has been observed in recent years that strict vaccination programs against *S. iniae* in aquaculture may have opened the door to increased infections by related pathogens such as *Streptococcus agalactiae*. To reduce the emergence of new pathogens that will fill the empty niche, a treatment needs to reduce the bacterial load to safe levels for the animals rather than removing the entire population altogether (Levin and Bull, 2004). Reduction of *S. iniae* rather than complete elimination may require some extra vigilance during certain

conditions, for example warm periods of heavy rainfall, perhaps in the form of an extra administration of bacteriocin or phage cocktail.

In conclusion, this study has identified bacteriophages and a bacteriocin with activity against *S. iniae*, and has opened the door for further research that can substantially increase our knowledge of the bacterium and our options for treating this aquatic pathogen in fish.

## LITERATURE CITED


Abutbul, S., Golan-Goldhirsh, A., Barazani, O. and Zilberg, D. (2004) Use of *Rosmarinus officinalis* as a treatment against *Streptococcus iniae* in tilapia (Oreochromis sp.). *Aquaculture* **238**: 97-105

Ackermann, H.-W. (2006a) Classification of Bacteriophages. In: R. Calendar (Ed), The Bacteriophages, Second Edition ed., pp. 8-16. Oxford University Press, Oxford.

Ackermann, H.W. (2001) Frequency of morphological phage descriptions in the year 2000. *Archives of Virology* **146**: 843-857

Ackermann, H.W. (2006b) Classification of Bacteriophages. In: R. Calendar (Ed), The Bacteriophages, pp. 8-16. Oxford University Press, Oxford.

Adams, M.H. (1959) Bacteriophages. Interscience publishers, London.

Alisky, J., Iczkowski, K., Rapoport, A. and Troitsky, N. (1998) Bacteriophages show promise as antimicrobial agents. *Journal of Infection* **36**: 5-15

Anon. (2006a) FDA Approval of Listeria-specific Bacteriophage Preparation on Ready-to-Eat (RTE) Meat and Poultry Products. In: F.C.f.F.S.A. Nutrition (Ed).

Anon. (2006b) OmniLytics Obtains First-Ever EPA Registration for Bacteriophage Usage.

Anon. (2007) Disease kills barramundi, Townsville Bulletin, Townsville, Queensland.

APVMA. (2010) Public Chemical Registration Information System. In: A.P.a.V.M. Authority (Ed). Vol. 2010. Commonwealth of Australia.

Austin, B. and Austin, D. (1999) Bacterial Fish Pathogens: Disease of Farmed and Wild Fish. 3 ed. Springer-Verlag, Chichester.

Aymerich, T., Holo, H., Havarstein, L.S., Hugas, M., Garriga, M. and Nes, I.F. (1996) Biochemical and genetic characterization of enterocin A from *Enterococcus faecium*, a new antilisterial bacteriocin in the pediocin family of bacteriocins. *Applied and Environmental Microbiology* **62**: 1676-1682

Bachrach, G., Zlotkin, A., Hurvitz, A., Evans, D.L. and Eldar, A. (2001) Recovery of *Streptococcus iniae* from diseased fish previously vaccinated with a *Streptococcus* vaccine. *Applied and Environmental Microbiology* **67**: 3756-3758

Baiano, J., Tumbol, R., Umapathy, A. and Barnes, A. (2008) Identification and molecular characterisation of a fibrinogen binding protein from *Streptococcus iniae*. *BMC Microbiology* **8**: 67

Barefoot, S.F. and Klaenhammer, T.R. (1983) Detection and activity of lactacin B, a bacteriocin produced by *Lactobacillus acidophilus*. *Applied and Environmental Microbiology* **45**: 1808-1815

Barksdale, L. and Arden, S.B. (1974) Persisting Bacteriophage Infections, Lysogeny, and Phage Conversions. *Annual Review of Microbiology* **28**: 265-300

Barnes, A.C., Horne, M.T. and Ellis, A.E. (2003a) Streptococcus iniae expresses a cell surface non-immune trout immunoglobulin-binding factor when grown in normal trout serum. *Fish & Shellfish Immunology* **15**: 425-431

Barnes, A.C., Young, F.M., Horne, M.T. and Ellis, A.E. (2003b) *Streptococcus iniae*: serological differences, presence of capsule and resistance to immune serum killing. *Diseases of Aquatic Organisms* **53**: 241-7

Barrow, P.A. and Soothill, J.S. (1997) Bacteriophage therapy and prophylaxis: rediscovery and renewed assessment of potential. *Trends in Microbiology* **5**: 268-271

Bercovier, H., Ghittino, C. and Eldar, A. (1997) Immunization with bacterial antigens: infections with streptococci and related organisms. *Developments in Biological Standardization* **90**: 153-160

Berridge, B.R., Fuller, J.D., de Azavedo, J., Low, D.E., Bercovier, H. and Frelier, P.F. (1998) Development of specific nested oligonucleotide PCR primers for the *Streptococcus iniae* 16S-23S ribosomal DNA intergenic spacer. *Journal of Clinical Microbiology* **36**: 2778-2781

Bhunia, A.K., Johnson, M.C. and Ray, B. (1988) Purification, characterization and antimicrobial spectrum of a bacteriocin produced by *Pediococcus acidilactici*. *Journal of Applied Microbiology* **65**: 261-268

Biswas, B., Adhya, S., Washart, P., Paul, B., Trostel, A.N., Powell, B., Carlton, R. and Merril, C.R. (2002) Bacteriophage therapy rescues mice bacteremic from a clinical isolate of vancomycin-resistant *Enterococcus faecium*. *Infection and Immunity* **70**: 204-210

Bolotin, S., Fuller, J.D., Bast, D.J., Beveridge, T.J. and de Azavedo, J.C.S. (2007) Capsule expression regulated by a two-component signal transduction system in *Streptococcus iniae*. *FEMS Immunology & Medical Microbiology* **50**: 366-374

Bonar, C. and Wagner, R. (2003) A third report of "golf ball disease" in an Amazon River dolphin (*Inia geoffrensis*) associated with *Streptococcus iniae*. *Journal of Zoo and Wildlife Medicine* **34**: 269-301

Bossi, L., Fuentes, J.A., Mora, G. and Figueroa-Bossi, N. (2003) Prophage contribution to bacterial population dynamics. *Journal of Bacteriology* **185**: 6467-6471

Bowser, P.R., Wooster, G.A., Getchell, R.G. and Timmons, M.B. (1998) *Streptococcus iniae* infection of tilapia *Oreochromis niloticus* in a recirculation production facility *Journal of the World Aquaculture Society* **29**: 335-339

Breitbart, M., Hewson, I., Felts, B., Mahaffy, J.M., Nulton, J., Salamon, P. and Rohwer, F. (2003) Metagenomic analyses of an uncultured viral community from human feces. *Journal of Bacteriology* **185**: 6220-6223

Broda, P. (1979) Plasmids. W. H. Freeman, Oxford & San Franciso.

Bromage, E.S. (1997) *Streptococcus iniae*, a bacterial infection in barramundi, *Lates calcarifer*. Honours thesis, James Cook University.

Bromage, E.S. and Owens, L. (2002) Infection of barramundi *Lates calcarifer* with *Streptococcus iniae*: effects of different routes of exposure. *Diseases of Aquatic Organisms* **52**: 199-205

Bromage, E.S. and Owens, L. (2009) Environmental factors affecting the susceptibility of barramundi to *Streptococcus iniae*. *Aquaculture* **290**: 224-228

Bromage, E.S., Thomas, J.A. and Owens, L. (1999) *Streptococcus iniae*, a bacterial infection in barramundi *Lates calcarifer*. *Diseases of Aquatic Organisms* **36**: 177-181

Brook, I. (1999) Bacterial Interference. *Critical Reviews in Microbiology* **25**: 155-172

Brunt, J. and Austin, B. (2005) Use of a probiotic to control lactococcosis and streptococcosis in rainbow trout, *Oncorhynchus mykiss* (Walbaum). *Journal of Fish Diseases* **28**: 693-701

Brussow, H., Canchaya, C. and Hardt, W.-D. (2004) Phages and the evolution of bacterial pathogens: from genomic rearrangements to lysogenic conversion. *Microbiology and Molecular Biology Reviews* **68**: 560-602

Buchanan, J.T., Colvin, K.M., Vicknair, M.R., Patel, S.K., Timmer, A.M. and Nizet, V. (2008) Strain-associated virulence factors of *Streptococcus iniae* in hybrid-striped bass. *Veterinary Microbiology* **131**: 145-153

Buchanan, J.T., Stannard, J.A., Lauth, X., Ostland, V.E., Powell, H.C., Westerman, M.E. and Nizet, V. (2005) *Streptococcus iniae* phosphoglucomutase is a virulence factor and a target for vaccine development. *Infection and Immunity* **73**: 6935-6944

Bull, J., Levin, B., DeRouin, T., Walker, N. and Bloch, C. (2002) Dynamics of success and failure in phage and antibiotic therapy in experimental infections. *BMC Microbiology* **2**: 35

Bundy, B.C., Franciszkowicz, M.J. and Swartz, J.R. (2008) *Escherichia coli*-based cell-free synthesis of virus-like particles. *Biotechnology and Bioengineering* **100**: 28-37

Busarcevic, M., Kojic, M., Dalgalarrondo, M., Chobert, J.M., Haertlé, T. and Topisirovic, L. (2008) Purification of bacteriocin LS1 produced by human oral isolate *Lactobacillus salivarius* BGHO1. *Oral Microbiology and Immunology* **23**: 254-258

Bushman, F. (2002) Lateral DNA transfer: mechanisms and consequences. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Campbell, A. (2003) The future of bacteriophage biology. *Nature Reviews Genetics* **4**: 471-477

Campos, C.A., Rodríguez, Ó., Calo-Mata, P., Prado, M. and Barros-Velázquez, J. (2006) Preliminary characterization of bacteriocins from *Lactococcus lactis*, *Enterococcus faecium* and *Enterococcus mundtii* strains isolated from turbot (*Psetta maxima*). *Food Research International* **39**: 356-364

Carlton, R.M., Noordman, W.H., Biswas, B., de Meester, E.D. and Loessner, M.J. (2005) Bacteriophage P100 for control of *Listeria monocytogenes* in foods: genome sequence, bioinformatic analyses, oral toxicity study, and application. *Regulatory Toxicology and Pharmacology* **43**: 301-312

Casjens, S. (2003) Prophages and bacterial genomics: what have we learned so far? *Molecular Microbiology* **49**: 277-300

Chen, J. and Novick, R.P. (2009) Phage-mediated intergeneric transfer of toxin genes. *Science* **323**: 139-141

Clark, J.R. and March, J.B. (2006) Bacteriophages and biotechnology: vaccines, gene therapy and antibacterials. *Trends in Biotechnology* **24**: 212-218

Colorni, A., Diamant, A., Eldar, A., Kvitt, H. and Zlotkin, A. (2002) *Streptococcus iniae* infections in Red Sea cage-cultured and wild fishes. *Diseases of Aquatic Organisms* **49**: 165-70

Cotter, P.D., Hill, C. and Ross, R.P. (2005) Bacteriocins: developing innate immunity for food. *Nature Reviews Microbiology* **3**: 777-788

Creeper, J.H. and Buller, N.B. (2006) An outbreak of *Streptococcus iniae* in barramundi (*Lates calcarifera*) in freshwater cage culture. *Australian Veterinary Journal* **84**: 408-411

Crothers-Stomps, C. (2007) Bacteriophage therapy for the biocontrol of *Vibrio harveyi* in the larval rearing system of the tropical rock lobster, *Panulirus ornatus*, James Cook University.

Crothers-Stomps, C., Høj, L., Bourne, D.G., Hall, M.R. and Owens, L. (2009) Isolation of lytic bacteriophage against *Vibrio harveyi*. *Journal of Applied Microbiology* **9999**

Crumplin, G.C. and Smith, J.T. (1981) The effect of R-factor plasmids on host-cell responses to nalidixic acid. I. Increased susceptibility of nalidixic acid-sensitive hosts. *Journal of Antimicrobial Chemotherapy* **7**: 379-388

Darwish, A.M. and Hobbs, M.S. (2005) Laboratory efficacy of amoxicillin for the control of *Streptococcus iniae* infection in blue tilapia *Journal of Aquatic Animal Health* **17**: 197-202

Darwish, A.M., Rawles, S. and Griffin, B. (2002) Dose titration of oxytetracycline against *Streptococcus iniae* infection in blue tilapia. International Aquatic Animal Health Symposium Proceedings.

Davidson, B.E., Powell, I.B. and Hillier, A.J. (1990) Temperate bacteriophages and lysogeny in lactic acid bacteria. *FEMS Microbiology Letters* **87**: 79-90

Daw, M.A. and Falkiner, F.R. (1996) Bacteriocins: nature, function and structure. *Micron* **27**: 467-479

Dawson, D.M.J. (2007) Lantibiotics as antimicrobial agents. *Expert Opinion on Therapeutic Patents* **17**: 365-369

Day, M. (2004) Bacterial sensitivity to bacteriophage in the aquatic environment. *Science Progress* **87**: 179-191

de Jong, A., van Hijum, S.A.F.T., Bijlsma, J.J.E., Kok, J. and Kuipers, O.P. (2006) BAGEL: a web-based bacteriocin genome mining tool. *Nucleic Acids Research* **34**: W273-279

de Kwaadsteniet, M., ten Doeschate, K. and Dicks, L.M.T. (2008) Characterization of the structural gene encoding Nisin F, a new lantibiotic produced by a *Lactococcus lactis* subsp. *lactis* isolate from freshwater catfish (*Clarias gariepinus*). *Applied and Environmental Microbiology* **74**: 547-549

De Vuyst, L. and Vandamme, E.J. (1994) Antimicrobial potential of lactic acid bacteria. In: L. De Vuyst and E.J. Vandamme (Eds), Bacteriocins of lactic acid bacteria: microbiology, genetics and applications, pp. 91-142. Blackie Academic, London, United Kingdom.

Delves-Broughton, J. (2005) Nisin as a food preservative. *Food Australia* **57**: 525-532

DePaola, A., Motes, M.L., Chan, A.M. and Suttle, C.A. (1998) Phages infecting *Vibrio vulnificus* are abundant and diverse in oysters (*Crassostrea virginica*) collected from the Gulf of Mexico. *Applied and Environmental Microbiology* **64**: 346-351

Douglas, J. (1975) Bacteriophages. Chapman and Hall, London.

Duckworth, D.H. (1976) "Who discovered bacteriophage?". *Microbiology and Molecular Biology Reviews* **40**: 793-802

Duffes, F., Leroi, F., Boyaval, P. and Dousset, X. (1999) Inhibition of *Listeria monocytogenes* by *Carnobacterium* spp. strains in a simulated cold smoked fish system stored at 4°C. *International Journal of Food Microbiology* **47**: 33-42

Eijsink, V., Axelsson, L., Diep, D., Håvarstein, L., Holo, H. and Nes, I. (2002) Production of class II bacteriocins by lactic acid bacteria; an example of biological warfare and communication. *Antonie van Leeuwenhoek* **81**: 639-654

Eldar, A., Bejerano, Y. and Bercovier, H. (1994) Streptococcus shiloi andStreptococcus difficile: Two new streptococcal species causing a meningoencephalitis in fish. *Current Microbiology* **28**: 139-143

Eldar, A., Frelier, P., Asanta, L., Varner, P., Lawhon, S. and Bercovier, H. (1995) *Streptococcus shiloi*, the name for an agent causing septicemic infection in fish, is a junior synonym of *Streptococcus iniae*. *International Journal of Systemic Bacteriology* **45**: 840-842

Eldar, A. and Ghittino, C. (1999) *Lactococcus garvieae* and *Streptococcus iniae* infections in rainbow trout *Oncorhynchus mykiss*: similar, but different diseases *Diseases of Aquatic Organisms* **36**: 227-231

Eldar, A., Hurvitz, A. and Bercovier, H. (1997a) Development and efficacy of a streptococcal vaccine for the Israeli trout farming. *Veterinary Immunology and Immunopathology* **56**: 175-183

Eldar, A., Lawhon, S., F. Frelier, P., Assenta, L., Simpson, B.R., Varner, P.W. and Bercovier, H. (1997b) Restriction fragment length polymorphisms of 16S rDNA and of whole rRNA genes (ribotyping) of *Streptococcus iniae* strains from the United States and Israel. *FEMS Microbiology Letters* **151**: 155-162

Elliman, J. (2006) Bacteriophage of *Burkholderia pseudomallei*; friend or foe?, James Cook University.

Evans, J.J., Klesius, P.H. and Shoemaker, C.A. (2006) Therapeutic and prophylactic immunization against *Streptococcus iniae* infection in hybrid striped bass (*Morone chrysops* X *Morone saxatilis*). *Aquaculture Research* **37**: 742-750

Evans, J.J., Shoemaker, C.A. and Klesius, P.H. (2000) Experimental *Streptococcus iniae* infection of hybrid striped bass (*Morone chrysops* X *Morone saxatalis*) and tilapia (*Oreochromis niloticus*) nare inoculation. *Aquaculture* **189**: 197-210

Evans, J.J., Shoemaker, C.A. and Klesius, P.H. (2001) Distribution of *Streptococcus iniae* in hybrid striped bass (*Morone chrysops* x *Morone saxatilis*) following nare inoculation. *Aquaculture* **194**: 233-243

Eyngor, M., Chilmonczyk, S., Zlotkin, A., Manuali, E., Lahav, D., Ghittino, C., Shapira, R., Hurvitz, A. and Eldar, A. (2007) Transcytosis of Streptococcus iniae through skin epithelial barriers: an in vitro study. *FEMS Microbiology Letters* **277**: 238-248

Eyngor, M., Tekoah, Y., Shapira, R., Hurvitz, A., Zlotkin, A., Lublin, A. and Eldar, A. (2008) Emergence of novel *Streptococcus iniae* exopolysaccharide-producing strains following vaccination with nonproducing strains. *Applied and Environmental Microbiology* **74**: 6892-6897

Facklam, R., Elliott, J., Shewmaker, L. and Reingold, A. (2005) Identification and characterization of sporadic isolates of *Streptococcus iniae* isolated from humans. *Journal of Clinical Microbiology* **43**: 933-937

Fang, T.J. and Yang, Y.S. (1995) Production and property of a bacteriocin-like inhibitor from *Lactococcus lactis* DY11212. *Food Science* **22**: 479-493

Ferguson, H.W., St John, V.S., Roach, C.J., Willoughby, S., Parker, C. and Ryan, R. (2000) Caribbean reef fish mortality associated with *Streptococcus iniae*. *The Veterinary Record* **147**: 662-664

Field, D., Connor, P.M.O., Cotter, P.D., Hill, C. and Ross, R.P. (2008) The generation of nisin variants with enhanced activity against specific Gram-positive pathogens. *Molecular Microbiology* **69**: 218-230

Foo, J.T.W., Ho, B. and Lam, T.J. (1985) Mass mortality in *Siganus canaliculatus* due to streptococcal infection. *Aquaculture* **49**: 185-195

Fuller, J.D., Bast, D.J., Nizet, V., Low, D.E. and de Azavedo, J.C.S. (2001) *Streptococcus iniae* virulence is associated with a distinct genetic profile. *Infection and Immunity* **69**: 1994-2000

Fuller, J.D., Camus, A.C., Duncan, C.L., Nizet, V., Bast, D.J., Thune, R.L., Low, D.E. and de Azavedo, J.C.S. (2002) Identification of a streptolysin S-associated gene cluster and its role in the pathogenesis of *Streptococcus iniae* disease. *Infection and Immunity* **70**: 5730-5739

Geis, A., Singh, J. and Teuber, M. (1983) Potential of lactic streptococci to produce bacteriocin. *Applied and Environmental Microbiology* **45**: 205-211

Ghittino, C., Latini, M., Agnetti, F., Panzieri, C., Lauro, L., Ciappelloni, R. and Petracca, G. (2003) Emerging pathologies in aquaculture: effects on production and food safety. *Veterinary Research Communications* **27**: 471-479

Gibson, G.R. and Roberfroid, M.B. (1995) Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. *Journal of Nutrition* **125**: 1401-1412

Gratia, A. (1925) Sur un remarquable example d'antagonisme entre deux souches de colibacille. *Comptes Rendus de la Société de Biologie* **93**: 1040-1041

Guinane, C.M., Cotter, P.D., Hill, C. and Ross, R.P. (2005) Microbial solutions to microbial problems; lactococcal bacteriocins for the control of undesirable biota in food. *Journal of Applied Microbiology* **98**: 1316-1325

Hagi, T. and Hoshino, T. (2009) Screening and characterization of potential probiotic lactic acid bacteria from cultured common carp intestine. *Bioscience, Biotechnology, and Biochemistry* **73**: 1479-1483

Hasper, H.E., Kramer, N.E., Smith, J.L., Hillman, J.D., Zachariah, C., Kuipers, O.P., de Kruijff, B. and Breukink, E. (2006) An Alternative Bactericidal Mechanism of Action for Lantibiotic Peptides That Target Lipid II. *Science* **313**: 1636-1637

Hermoso, J.A., Garcia, J.L. and Garcia, P. (2007) Taking aim on bacterial pathogens: from phage therapy to enzybiotics. *Current Opinion in Microbiology* **10**: 461-472

Hoover, D.G. and Harlander, S.K. (1993) Screening methods for detecting bacteriocin activity. In: D.G. Hoover and L.R. Steenson (Eds), Bacteriocins of lactic acid bacteria. Academic Press, San Diego.

ICTV. (2005) Virus Taxonomy. Classification and Nomenclature of Viruses, Eighth Report of the International Committee on the Taxonomy of Viruses, edited by C.M. Fauquet, M.A. Mayo, J. Maniloff, U. Desselberger and L.A. Ball. Elsevier, San Diego.

ICTVdB_Management. (2006) Siphoviridae. In: C. Buchen-Osmond (Ed), *ICTVdB - The Universal Virus Database*, version 3. Columbia University, New York.

Imbeault, S., Parent, S., Lagace, M., Uhland, C.F. and Blais, J.F. (2006) Using bacteriophages to prevent furunculosis caused by *Aeromonas salmonicida* in farmed brook trout. *Journal of Aquatic Animal Health* **18**: 203-214

Iwamoto, E., Matsuoka, S., Ishida, M., Miyoshi, Y., Park, S.C., Yoshida, T. and Nakai, T. (2008) Phage therapy against *Streptococcus iniae* infection of Japanese flounder *Paralichtys olivaceus*. The 7th Symposium on Diseases in Asian Aquaculture.

Jack, R.W., Tagg, J.R. and Ray, B. (1995) Bacteriocins of gram-positive bacteria. *Microbiology and Molecular Biology Reviews* **59**: 171-200

Jacob, F., Siminovitch, L. and Wollman, E. (1952) Sur la biosynthese d'une colicine et sur son mode d'action. **83**: 295-315

Jensen, E.C., Schrader, H.S., Rieland, B., Thompson, T.L., Lee, K.W., Nickerson, K.W. and Kokjohn, T.A. (1998) Prevalence of broad-host-range lytic bacteriophages of *Sphaerotilus natans*, *Escherichia coli*, and *Pseudomonas aeruginosa*. *Applied and Environmental Microbiology* **64**: 575-580

Jiang, S.C. and Paul, J.H. (1998) Significance of lysogeny in the marine environment: studies with isolates and a model of lysogenic phage production. *Microbial Ecology* **35**: 235-243

Johnson, R.P., Gyles, C.L., Huff, W.E., Ojha, S., Huff, G.R., Rath, N.C. and Donoghue, A.M. (2008) Bacteriophages for prophylaxis and therapy in cattle, poultry and pigs. *Animal Health Research Reviews* **9**: 201-215

Karunasagar, I., Shivu, M.M., Girisha, S.K., Krohne, G. and Karunasagar, I. (2007) Biocontrol of pathogens in shrimp hatcheries using bacteriophages. *Aquaculture* **268**: 288-292

Kasuda, R. and Salati, F. (1999) *Enterococcus seriolicida* and *Streptococcus iniae*. In: P.T.K. Woo and D.W. Bruno (Eds), Fish Diseases and Disorders, pp. 303-317. Vol. 3. CABI Publishing, Wallingford.

Kim, D.H. and Austin, B. (2008) Characterization of probiotic carnobacteria isolated from rainbow trout *Oncorhynchus mykiss* intestine. *Letters in Applied Microbiology* **47**: 141-147

Kim, J.H., Gomez, D.K., Choresca Jr, C.H. and Park, S.C. (2007a) Detection of major bacterial and viral pathogens in trash fish used to feed cultured flounder in Korea. *Aquaculture* **272**: 105-110

Kim, M.S., Choi, S.H., Lee, E.H., Nam, Y.K., Kim, S.K. and Kim, K.H. (2007b) [alpha]-enolase, a plasmin(ogen) binding and cell wall associating protein from a fish pathogenic *Streptococcus iniae* strain. *Aquaculture* **265**: 55-60

Kitao, T., Aoki, T. and Sakoh, R. (1981) Epizootic caused by beta-haemolytic *Streptococcus* species in cultured freshwater fish. *Fish Pathology* **15**: 301-307

Klaenhammer, T.R. (1988) Bacteriocins of lactic acid bacteria. *Biochimie* **70**: 337-349

Klesius, P., Evans, J., Shoemaker, C., Yeh, H., Goodwin, A.E., Adams, A. and Thompson, K. (2006) Rapid detection and identification of Streptococcus iniae using a monoclonal antibody-based indirect fluorescent antibody technique. *Aquaculture* **258**: 180-186

Klesius, P.H., Evans, J.J. and Shoemaker, C.A. (2007) The macrophage chemotactic activity of *Streptococcus agalactiae* and *Streptococcus iniae* extracellular products (ECP). *Fish & Shellfish Immunology* **22**: 443-450

Kobayashi, S., Hirakura, Y. and Matsuzaki, K. (2001) Bacteria-selective synergism between the antimicrobial peptides α-helical magainin 2 and cyclic β-sheet tachyplesin I: toward cocktail therapy. *Biochemistry* **40**: 14330-14335

Kropinski, A.M., Prangishvili, D. and Lavigne, R. (2009) Position paper: the creation of a rational scheme for the nomenclature of viruses of *Bacteria* and *Archaea*. *Environmental Microbiology* **11**: 2775-2777

Kvitt, H. and Colorni, A. (2004) Strain variation and geographic endemism in *Streptococcus iniae*. *Diseases of Aquatic Organisms* **61**: 67-73

Labrie, S. and Moineau, S. (2000) Multiplex PCR for detection and identification of Lactococcal bacteriophages. *Applied and Environmental Microbiology* **66**: 987-994

Lahav, D., Eyngor, M., Hurvitz, A., Ghittino, C., Lublin, A. and Eldar, A. (2004) *Streptococcus iniae* type II infections in rainbow trout *Oncorhynchus mykiss*. *Diseases of Aquatic Organisms* **62**: 177-180

Lan, C.Q., Oddone, G., Mills, D.A. and Block, D.E. (2006) Kinetics of *Lactococcus lactis* growth and metabolite formation under aerobic and anaerobic conditions in the presence or absence of hemin. *Biotechnology and Bioengineering* **95**: 1070-1080

Lau, S.K.P., Woo, P.C.Y., Luk, W.-k., Fung, A.M.Y., Hui, W.-t., Fong, A.H.C., Chow, C.-w., Wong, S.S.Y. and Yuen, K.-y. (2006) Clinical isolates of *Streptococcus iniae* from Asia are more mucoid and [beta]-hemolytic than those from North America. *Diagnostic Microbiology and Infectious Disease* **54**: 177-181

Lau, S.K.P., Woo, P.C.Y., Tse, H., Leung, K.-W., Wong, S.S.Y. and Yuen, K.-Y. (2003) Invasive *Streptococcus iniae* infections outside North America. *Journal of Clinical Microbiology* **41**: 1004-1009

Levin, B.R. and Bull, J.J. (2004) Population and evolutionary dynamics of phage therapy. *Nature Reviews Microbiology* **2**: 166-173

Levin, B.R., Stewart, F.M. and Chao, L. (1977) Resource-limited growth, competition, and predation: a model and experimental studies with bacteria and bacteriophage. *American Naturalist* **111**: 3

Li, P. and Gatlin, D.M. (2004) Dietary brewers yeast and the prebiotic Grobiotic(TM)AE influence growth performance, immune responses and resistance of hybrid striped bass (*Morone chrysops* x *M. saxatilis*) to *Streptococcus iniae* infection. *Aquaculture* **231**: 445-456

Li, P., Wang, X. and Gatlin Iii, D.M. (2006) Evaluation of levamisole as a feed additive for growth and health management of hybrid striped bass (*Morone chrysops* x *Morone saxatilis*). *Aquaculture* **251**: 201-209

Locke, J.B., Aziz, R.K., Vicknair, M.R., Nizet, V. and Buchanan, J.T. (2008) *Streptococcus iniae* M-like protein contributes to virulence in fish and is a target for live attenuated vaccine development. *PLoS ONE* **3**: e2824

Locke, J.B., Colvin, K.M., Datta, A.K., Patel, S.K., Naidu, N.N., Neely, M.N., Nizet, V. and Buchanan, J.T. (2007a) *Streptococcus iniae* capsule impairs phagocytic clearance and contributes to virulence in fish. *Journal of Bacteriology* **189**: 1279-1287

Locke, J.B., Colvin, K.M., Varki, N., Vicknair, M.R., Nizet, V. and Buchanan, J.T. (2007b) *Streptococcus iniae* beta-hemolysin streptolysin S is a virulence factor in fish infection. *Diseases of Aquatic Organisms* **76**: 17-26

Loessner, M.J., Goeppl, S. and Busse, M. (1991) Comparative inducibility of bacteriophage in naturally lysogenic and lysogenized strains of *Listeria* spp. by u.v. light and Mitomycin C. *Letters in Applied Microbiology* **12**: 196-199

Lowe, B.A., Miller, J.D. and Neely, M.N. (2007) Analysis of the polysaccharide capsule of the systemic pathogen, *Streptococcus iniae* and its implications in virulence. *Infection and Immunity* **75**: 1255-1264

Lu, T.K. and Collins, J.J. (2009) Engineered bacteriophage targeting gene networks as adjuvants for antibiotic therapy. *Proceedings of the National Academy of Sciences* **106**: 4629-4634

Maloy, S.R., Cronan, J.E. and Freifelder, D. (1994) Microbial Genetics. 2 ed. Jones and Bartlett Publishers.

Martinez-Cuesta, M.C., Kok, J., Herranz, E., Pelaez, C., Requena, T. and Buist, G. (2000) Requirement of autolytic activity for bacteriocin-induced lysis. *Applied and Environmental Microbiology* **66**: 3174-3179

Mata, A.I., Blanco, M.M., Dominguez, L., Fernandez-Garayzabal, J.F. and Gibello, A. (2004a) Development of a PCR assay for *Streptococcus iniae* based on the lactate oxidase (lctO) gene with potential diagnostic value. *Veterinary Microbiology* **101**: 109-116

Mata, A.I., Gibello, A., Casamayor, A., Blanco, M.M., Dominguez, L. and Fernandez-Garayzabal, J.F. (2004b) Multiplex PCR assay for detection of bacterial pathogens associated with warm-water streptococcosis in fish. *Applied and Environmental Microbiology* **70**: 3183-3187

Matsuoka, S., Hashizume, T., Kanzaki, H., Iwamoto, E., Park, S.C., Yoshida, T. and Nakai, T. (2007) Phage therapy against β-hemolytic Streptococcicosis of Japanese Flounder *Paralichthys olivaceus*. *Fish Pathology* **42**: 181-189

Matsuzaki, S., Rashel, M., Uchiyama, J., Sakurai, S., Ujihara, T., Kuroda, M., Ikeuchi, M., Tani, T., Fujieda, M., Wakiguchi, H. and Imai, S. (2005) Bacteriophage therapy: a revitalized therapy against bacterial infectious diseases. *Journal of Infection and Chemotherapy* **11**: 211-219

Matsuzaki, S., Yasuda, M., Nishikawa, H., Kuroda, M., Ujihara, T., Shuin, T., Shen, Y., Jin, Z., Fujimoto, S., Nasimuzzaman, M.Â D., Wakiguchi, H., Sugihara, S., Sugiura, T., Koda, S., Muraoka, A. and Imai, S. (2003) Experimental protection of mice against lethal *Staphylococcus aureus* Infection by novel bacteriophage Ï†MR11. *The Journal of Infectious Diseases* **187**: 613-624

Mattey, M. and Spencer, J. (2008) Bacteriophage therapy -- cooked goose or Phoenix rising? *Current Opinion in Biotechnology* **19**: 608-612

Mauel, M.J., Miller, D.L., Frazier, K.S. and Hines, M.E., 2nd. (2002) Bacterial pathogens isolated from cultured bullfrogs (*Rana castesbeiana*). *Journal of Veterinary Diagnostic Investigation* **14**: 431-433

McAuliffe, O., Hill, C. and Ross, R.P. (2000) Each peptide of the two-component lantibiotic lacticin 3147 requires a separate modification enzyme for activity. *Microbiology* **146**: 2147-2154

McAuliffe, O., Ross, R.P. and Fitzgerald, G.F. (2007) The new phage biology: from genomics to applications. In: S. McGrath and D. van Sinderen (Eds), Bacteriophage: Genetics and Molecular Biology. Caister Academic.

McNulty, S.T., Klesius, P.H., Shoemaker, C.A. and Evans, J.J. (2003) *Streptococcus iniae* infection and tissue distribution in hybrid striped bass (*Morone chrysops* x *Morone saxatilis*) following inoculation of the gills. *Aquaculture* **220**: 165-173

Melancon, D. and Grenier, D. (2003) Production and properties of bacteriocin-like inhibitory substances from the swine pathogen *Streptococcus suis* serotype 2. *Applied and Environmental Microbiology* **69**: 4482-4488

Mendez, J., Jofre, J., Lucena, F., Contreras, N., Mooijman, K. and Araujo, R. (2002) Conservation of phage reference materials and water samples containing bacteriophages of enteric bacteria. *Journal of Virological Methods* **106**: 215-224

Merril, C.R., Biswas, B., Carlton, R., Jensen, N.C., Creed, G.J., Zullo, S. and Adhya, S. (1996) Long-circulating bacteriophage as antibacterial agents. *Proceedings of the National Academy of Sciences* **93**: 3188-3192

Michel, C., Pelletier, C., Boussaha, M., Douet, D.-G., Lautraite, A. and Tailliez, P. (2007) Diversity of Lactic Acid Bacteria Associated with Fish and the Fish Farm Environment, Established by Amplified rRNA Gene Restriction Analysis. *Applied and Environmental Microbiology* **73**: 2947-2955

Miller, J.D. and Neely, M.N. (2005) Large-scale screen highlights the importance of capsule for virulence in the zoonotic pathogen *Streptococcus iniae*. *Infection and Immunity* **73**: 921-934

Ming, X. and Daeschel, M.A. (1993) Nisin resistance of foodborne bacteria and the specific resistance responses of *Listeria monocytogenes* Scott A *Journal of Food Protection* **56**: 944-948

Moreno, I., Lerayer, A.L.S., Baldini, V.L.S. and Leitao, M.F.d.F. (2000) Characterization of bacteriocins produced by *Lactococcus lactis* strains. *Brazilian Journal of Microbiology* **31**

Morgan, S., Ross, R.P. and Hill, C. (1995) Bacteriolytic activity caused by the presence of a novel lactococcal plasmid encoding lactococcins A, B, and M. *Applied and Environmental Microbiology* **61**: 2995-3001

Morrison, S. and Rainnie, D.J. (2004) Bacteriophage therapy: an alternative to antibiotic therapy in aquaculture? .Canadian Technical Report of Fisheries and Aquatic Sciences 2532: v + 23p.

Mortvedt-Abildgaard, C.I., Nissen-Meyer, J., Jelle, B., Grenov, B., Skaugen, M. and Nes, I.F. (1995) Production and pH-Dependent Bactericidal Activity of Lactocin S, a Lantibiotic from *Lactobacillus sake* L45. *Applied and Environmental Microbiology* **61**: 175-179

Nakai, T. and Park, S.C. (2002) Bacteriophage therapy of infectious diseases in aquaculture. *Research in Microbiology* **153**: 13-18

Nakai, T., Sugimoto, R., Park, K.H., Matsuoka, S., Mori, K., Nishioka, T. and Maruyama, K. (1999) Protective effects of bacteriophage on experimental *Lactococcus garvieae* infection in yellowtail. *Diseases of Aquatic Organisms* **37**: 33-41

Nawawi, R.A., Baiano, J. and Barnes, A.C. (2008) Genetic variability amongst Streptococcus iniae isolates from Australia. *Journal of Fish Diseases* **31**: 305-309

Nawawi, R.A., Baiano, J.C.F., Kvennefors, E.C.E. and Barnes, A.C. (2009) Host-Directed Evolution of a Novel Lactate Oxidase in *Streptococcus iniae* Isolates from Barramundi (*Lates calcarifer*). *Applied and Environmental Microbiology* **75**: 2908-2919

Neely, M.N., Pfeifer, J.D. and Caparon, M. (2002) Streptococcus-zebrafish model of bacterial pathogenesis. *Infection and Immunity* **70**: 3904-3914

Nes, I.F., Diep, D.B. and Holo, H. (2007) Bacteriocin Diversity in Streptococcus and Enterococcus. *Journal of Bacteriology* **189**: 1189-1198

Neve, H., Geis, A. and Teuber, M. (1984) Conjugal transfer and characterization of bacteriocin plasmids in group N (lactic acid) streptococci. *Journal of Bacteriology* **157**: 833-838

Nguyen, H.T. and Kanai, K. (1999) Selective agars for the isolation of *Streptococcus iniae* from Japanese flounder, *Paralichthys olivaceus*, and its cultural environment. *Journal of Applied Microbiology* **86**: 769-776

Nguyen, H.T., Kanai, K. and Yoshikoshi, K. (2002) Ecological investigation of *Streptococcus iniae* in cultured Japanese flounder (*Paralichthys olivaceus*) using selective isolation procedures. *Aquaculture* **205**: 7-17

Nho, S.-W., Shin, G.-W., Park, S.-B., Jang, H.-B., Jang, Cha, I.-S., Ha, M.-A., Kim, Y.-R., Park, Y.-K., Dalvi, R.S., Kang, B.-J., Joh, S.-J. and Jung, T.-S. (2009) Phenotypic characteristics of *Streptococcus iniae* and *Streptococcus parauberis* isolated from olive flounder (*Paralichthys olivaceus*). *FEMS Microbiology Letters* **293**: 20-27

Oakey, H.J. and Owens, L. (2000) A new bacteriophage, VHML, isolated from a toxin-producing strain of Vibrio harveyi in tropical Australia. *Journal of Applied Microbiology* **89**: 702-709

Owens, L. Personal Communication, September 2007.

Parisien, A., Allain, B., Zhang, J., Mandeville, R. and Lan, C.Q. (2008) Novel alternatives to antibiotics: bacteriophages, bacterial cell wall hydrolases, and antimicrobial peptides. *Journal of Applied Microbiology* **104**: 1-13

Park, K.H., Matsuoka, S., Nakai, T. and Muroga, K. (1997) A virulent bacteriophage of *Lactococus garvieae* (formerly *Enterococcus seriolicida*) isolated from yellowtail *Seriola quinqueradiata*. *Diseases of Aquatic Organisms* **29**: 145-149

Park, S.C. and Nakai, T. (2003) Bacteriophage control of *Pseudomonas plecoglossicida* infection in ayu *Plecoglossus altivelis*. *Diseases of Aquatic Organisms* **53**: 33-39

Park, S.C., Shimamura, I., Fukunaga, M., Mori, K.I. and Nakai, T. (2000) Isolation of bacteriophages specific to a fish pathogen, *Pseudomonas plecoglossicida*, as a candidate for disease control. *Applied and Environmental Microbiology* **66**: 1416-1422

Park, Y.-K., Nho, S.-W., Shin, G.-W., Park, S.-B., Jang, H.-B., Cha, I.-S., Ha, M.-A., Kim, Y.-R., Dalvi, R.S., Kang, B.-J. and Jung, T.-S. (2009) Antibiotic susceptibility and resistance of Streptococcus iniae and Streptococcus parauberis isolated from olive flounder (Paralichthys olivaceus). *Veterinary Microbiology* **136**: 76-81

Paterson, W.D., Douglas, R.J., Grinyer, I. and McDermott, L.A. (1969) Isolation and preliminary characterization of some *Aeromonas salmonicida* bacteriophages. *Journal of the Fisheries Research Board of Canada* **26**: 629-632

Paul, J.H., Sullivan, M.B., Segall, A.M. and Rohwer, F. (2002) Marine phage genomics. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* **133**: 463-476

Perera, R.P., Johnson, S.K. and Lewis, D.H. (1997) Epizootiological aspects of *Streptococcus iniae* affecting tilapia in Texas. *Aquaculture* **152**: 25-33

Peres, H., Lim, C. and Klesius, P.H. (2004) Growth, chemical composition and resistance to *Streptococcus iniae* challenge of juvenile Nile tilapia (*Oreochromis niloticus*) fed graded levels of dietary inositol. *Aquaculture* **235**: 423-432

Petty, N.K., Evans, T.J., Fineran, P.C. and Salmond, G.P.C. (2007) Biotechnological exploitation of bacteriophage research. *Trends in Biotechnology* **25**: 7-15

Pier, G. and Madin, S. (1976) *Streptococcus iniae* sp. nov., a beta-hemolytic streptococcus isolated from an Amaxon freshwater dolphin, *Inia geoffrensis*. *International Journal of Systemic Bacteriology* **26**: 545-553

Pier, G., Madin, S. and Al-Nakeeb, S. (1978) Isolation and characterization of a second isolate of *Streptococcus iniae*. *International Journal of Systemic Bacteriology* **28**: 311-314

Pilet, M.-F., Dousset, X., Barr, Rachel, Novel, G., Desmazeaud, M. and Piard, J.-C. (1995) Evidence for two bacteriocins produced by *Carnobacterium piscicola* and *Carnobacterium divergens* isolated from fish and active against *Listeria monocytogenes*. *Journal of Food Protection* **58**: 256-262

Pingitore, E.V., Salvucci, E., Sesma, F. and Nader-Macias, M.E. (2007) Different strategies for purification of antimicrobial peptides from Lactic Acid Bacteria (LAB). In: A. Mendez-Vilas (Ed), Communicating Current Research and Educational Topics and Trends in Applied Microbiology, pp. 557-568. Vol. 1. Formatex.

Ramirez, M., Severina, E. and Tomasz, A. (1999) A high incidence of prophage carriage among natural isolates of *Streptococcus pneumoniae*. *Journal of Bacteriology* **181**: 3618-3625

Reddy, S.T., Swartz, M.A. and Hubbell, J.A. (2006) Targeting dendritic cells with biomaterials: developing the next generation of vaccines. *Trends in Immunology* **27**: 573-579

Reid, G., Howard, J. and Gan, B.S. (2001) Can bacterial interference prevent infection? *Trends in Microbiology* **9**: 424-428

Reyrolle, J., Chopin, M.C., Letellier, F. and Novel, G. (1982) Lysogenic strains of lactic acid streptococci and lytic spectra of their temperate bacteriophages. *Applied and Environmental Microbiology* **43**: 349-356

Riley, M.A. and Wertz, J.E. (2002) Bacteriocins: evolution, ecology, and application. *Annual Review of Microbiology* **56**: 117-137

Roach, J.C.M., Levett, P.N. and Lavoie, M.C. (2006) Identification of *Streptococcus iniae* by commercial bacterial identification systems. *Journal of Microbiological Methods* **67**: 20-26

Roberts, R.J. (2001) Fish Pathology. 3 ed. Saunders, Philadelphia.

Robertsen, B., Roerstad, G., Engstad, R. and Raa, J. (1990) Enhancement of non-specific disease resistance in Atlantic salmon, *Salmo salar* L., by a glucan from *Saccharomyces cerevisiae* cell walls. *Journal of Fish Diseases* **13**: 391-400

Rodríguez, E., González, B., Gaya, P., Nuñez, M. and Medina, M. (2000) Diversity of bacteriocins produced by lactic acid bacteria isolated from raw milk. *International Dairy Journal* **10**: 7-15

Rodríguez, J.M., Cintas, L.M., Casaus, P., Horn, N., Dodd, H.M., Hernandez, P.E. and Gasson, M.J. (1995) Isolation of nisin-producing *Lactococcus lactis* strains from dry fermented sausages. *Journal of Applied Microbiology* **78**: 109-115

Rogers, L.A. (1928) Limiting factors in the lactic acid fermentation. *Journal of Bacteriology* **16**: 321

Russo, R., Yanong, R.P.E. and Mitchell, H. (2006) Dietary beta-glucans and nucleotides enhance resistance of red-tail black shark (*Epalzeorhynchos bicolor*, fam.

Cyprinidae) to *Streptococcus iniae* infection. *Journal of the World Aquaculture Society* **37**: 298-306

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Extraction of bacteriophage Lambda DNA. In: N. Ford (Ed), Molecular Cloning: a laboratory manual, 2 ed., pp. 2.80-2.81. Cold Spring Harbor Laboratory Press, New York.

Sanger, F., Coulson, A.R., Friedmann, T., Air, G.M., Barrell, B.G., Brown, N.L., Fiddes, J.C., Hutchison, C.A., Slocombe, P.M. and Smith, M. (1978) The nucleotide sequence of bacteriophage [phi]X174. *Journal of Molecular Biology* **125**: 225-246

Seeley, N.D. and Primrose, S.B. (1982) A review: the isolation of bacteriophages from the environment. *Journal of Applied Microbiology* **53**: 1-17

Seng, L. and Colorni, A. (2002) Infectious diseases of warmwater fish in marine and brackish waters. In: P.T.K. Woo, D.W. Bruno and L.H.S. Lim (Eds), Diseases and Disorders of Finfish in Cage Culture, pp. 193-230. CABI Publishing, Wallingford.

Serwer, P., Hayes, S.J., Thomas, J.A., Griess, G.A. and Hardies, S.C. (2007) Rapid determination of genomic DNA length for new bacteriophages. *Electrophoresis* **28**: 1896-1902

Shehane, S.D. and Sizemore, R.K. (2002) Isolation and preliminary characterization of bacteriocins produced by *Vibrio vulnificus*. *Journal of Applied Microbiology* **92**: 322-328

Shin, G.W., Palaksha, K.J., Yang, H.H., Shin, Y.S., Kim, Y.R., Lee, E.Y., Oh, M.J. and Jung, T.S. (2006) Partial two-dimensional gel electrophoresis (2-DE) maps of *Streptococcus iniae* ATCC29178 and *Lactococcus garvieae* KG9408. *Diseases of Aquatic Organisms* **70**: 71-79

Shoemaker, C.A., Evans, J.J. and Klesius, P.H. (2000) Density and dose: factors affecting mortality of *Streptococcus iniae* infected tilapia (*Oreochromis niloticus*). *Aquaculture* **188**: 229-235

Skurnik, M., Pajunen, M. and Kiljunen, S. (2007) Biotechnological challenges of phage therapy. *Biotechnology Letters* **29**: 995-1003

Skurnik, M. and Strauch, E. (2006) Phage therapy: facts and fiction. *International Journal of Medical Microbiology* **296**: 5-14

Smith, H.W. and Huggins, M.B. (1982) Successful treatment of experimental *Escherichia coli* infections in mice using phage: its general superiority over antibiotics. *Journal of General Microbiology* **128**: 307-318

Smith, H.W. and Huggins, M.B. (1983) Effectiveness of phages in treating experimental *Escherichia coli* diarrhoea in calves, piglets and lambs. *Journal of General Microbiology* **129**: 2659-2675

Smith, H.W., Huggins, M.B. and Shaw, K.M. (1987) Factors influencing the survival and multiplication of bacteriophages in calves and in their environment. *Journal of General Microbiology* **133**: 1127-1135

Smithyman, T. Personal Communication, April 2009.

Spellberg, B., Powers, JohnÂ H., Brass, EricÂ P., Miller, LorenÂ G. and Edwards, J.JohnÂ E. (2004) Trends in antimicrobial drug development: implications for the future. *Clinical Infectious Diseases* **38**: 1279-1286

Stanley, E., Fitzgerald, G.F., Le Marrec, C., Fayard, B. and van Sinderen, D. (1997) Sequence analysis and characterization of phi O1205, a temperate bacteriophage infecting *Streptococcus thermophilus* CNRZ1205. *Microbiology* **143**: 3417-3429

Stenholm, A.R., Dalsgaard, I. and Middelboe, M. (2008) Isolation and Characterization of Bacteriophages Infecting the Fish Pathogen *Flavobacterium psychrophilum*. *Applied and Environmental Microbiology* **74**: 4070-4078

Stent, G.S. (1963) Molecular Biology of Bacterial Viruses. Freeman, San Francisco.

Stoffels, G., Nes, I.F. and Guomundsdóttir, A. (1992) Isolation and properties of a bacteriocin-producing *Carnobacterium piscicola* isolated from fish. *Journal of Applied Microbiology* **73**: 309-316

Stoffregen, D.A., Backman, S.C., Perham, R.E., Bowser, P.R. and Babish, J.G. (1996) Initial disease report of *Streptococcus iniae* infection in hybrid striped (sunshine) bass and successful therapeutic intervention with the fluoroquinolone antibacterial enrofloxacin *Journal of the World Aquaculture Society* **27**: 420-434

Stoskopf, M. (1993) Fish Medicine. W.B. Saunders Company, Philidelphia.

Strauch, E., Kaspar, H., Schaudinn, C., Dersch, P., Madela, K., Gewinner, C., Hertwig, S., Wecke, J. and Appel, B. (2001) Characterization of enterocoliticin, a phage tail-like bacteriocin, and its effect on pathogenic *Yersinia enterocolitica* strains. *Applied and Environmental Microbiology* **67**: 5634-5642

Summers, W.C. (2001) Bacteriophage therapy. *Annual Review of Microbiology* **55**: 437-451

Sun, J.-R., Yan, J.-C., Yeh, C.-Y., Lee, S.-Y. and Lu, J.-J. (2007) Invasive infection with Streptococcus iniae in Taiwan. *Journal of Medical Microbiology* **56**: 1246-1249

Tagg, J.R., Dajani, A.S. and Wannamaker, L.W. (1976) Bacteriocins of gram-positive bacteria. *Bacteriological Reviews* **40**: 722-756

Tagg, J.R. and McGiven, A.R. (1971) Assay System for Bacteriocins. *Applied and Environmental Microbiology* **21**: 943-

Twort, F.W. (1915) An investigation on the nature of ultra-microscopic viruses. *The Lancet* **186**: 1241-1243

Ventura, M., Bruttin, A., Canchaya, C. and Brüssow, H. (2002) Transcription analysis of *Streptococcus thermophilus* phages in the lysogenic state. *Virology* **302**: 21-32

Verner-Jeffreys, D.W., Algoet, M., Pond, M.J., Virdee, H.K., Bagwell, N.J. and Roberts, E.G. (2007) Furunculosis in Atlantic salmon (*Salmo salar* L.) is not readily controllable by bacteriophage therapy. *Aquaculture* **270**: 475-484

Vignolo, G., Palacios, J., Farías, M.E., Sesma, F., Schillinger, U., Holzapfel, W. and Oliver, G. (2000) Combined effect of bacteriocins on the survival of various *Listeria* species in broth and meat system. *Current Microbiology* **41**: 410-416

Vinod, M.G., Shivu, M.M., Umesha, K.R., Rajeeva, B.C., Krohne, G., Karunasagar, I. and Karunasagar, I. (2006) Isolation of Vibrio harveyi bacteriophage with a potential for biocontrol of luminous vibriosis in hatchery environments. *Aquaculture* **255**: 117-124

Walakira, J.K., Carrias, A.A., Hossain, M.J., Jones, E., Terhune, J.S. and Liles, M.R. (2008) Identification and characterization of bacteriophages specific to the catfish pathogen, *Edwardsiella ictaluri*. *Journal of Applied Microbiology* **105**: 2133-2142

Weber-Dabrowska, B., Mulczyk, M. and Gorski, A. (2001) Bacteriophage therapy for infections in cancer patients. *Clinical and Applied Immunology Reviews* **1**: 131-134

Weinstein, M.R., Litt, M., Kertesz, D.A., Wyper, P., Rose, D., Coulter, M., McGeer, A., Facklam, R., Ostach, C., Willey, B.M., Borczyk, A., Low, D.E. and The, S.i.S.G. (1997) Invasive infections due to a fish pathogen, *Streptococcus iniae*. *New England Journal of Medicine* **337**: 589-594

Westwater, C., Kasman, L.M., Schofield, D.A., Werner, P.A., Dolan, J.W., Schmidt, M.G. and Norris, J.S. (2003) Use of genetically engineered phage to deliver antimicrobial agents to bacteria: an alternative therapy for treatment of bacterial tnfections. *Antimicrobial Agents and Chemotherapy* **47**: 1301-1307

Whittington, R., Lim, C. and Klesius, P.H. (2005) Effect of dietary [beta]-glucan levels on the growth response and efficacy of *Streptococcus iniae* vaccine in Nile tilapia, *Oreochromis niloticus*. *Aquaculture* **248**: 217-225

Wirawan, R.E., Klesse, N.A., Jack, R.W. and Tagg, J.R. (2006) Molecular and genetic characterization of a novel nisin variant produced by *Streptococcus uberis*. *Applied and Environmental Microbiology* **72**: 1148-1156

Wooley, R.E., Ritchie, B.W. and Burnley, V.V. (2004) *In vitro* effect of a buffered chelating agent and neomycin or oxytetracycline on bacteria associated with diseases of fish. *Diseases of Aquatic Organisms* **59**: 263-267

Wu, J.L. and Chao, W.J. (1982) Isolation and application of a new bacteriophage, ϕET-1, which infect *Edwardsiella tarda*, the pathogen of edwardsiellosis. *Fish Disease Research* **IV**: 8-17

Yamazaki, K., Suzuki, M., Kawai, Y., Inque, N. and Montville, T. (2003) Inhibition of *Listeria monocytogenes* in cold-smoked salmon by *Carnobacterium piscicola* CS526 isolated from frozen surimi. *Journal of Food Protection* **66**: 1420-1425

Yanong, R.P.E., Curtis, E.W., Simmons, R., Bhattaram, V.A., Gopalakrishnan, M., Ketabi, N., Nagaraja, N.V. and Derendorf, H. (2005) Pharmacokinetic studies of florfenicol in koi carp and threespot gourami *Trichogaster trichopterus* after oral and intramuscular treatment. *Journal of Aquatic Animal Health* **17**: 129-137

Zlotkin, A., Chilmonczyk, S., Eyngor, M., Hurvitz, A., Ghittino, C. and Eldar, A. (2003) Trojan horse effect: phagocyte-mediated *Streptococcus iniae* infection of fish. *Infection and Immunity* **71**: 2318-2325

Zlotkin, A., Hershko, H. and Eldar, A. (1998) Possible transmission of *Streptococcus iniae* from wild fish to cultured marine fish. *Applied and Environmental Microbiology* **64**: 4065-4067

## APPENDIX 1

## BUFFERS, SOLUTIONS AND AGARS

### 1.1 Tris HCl (1M)

| | |
|---|---|
| Tris | 12.14 g |
| Double distilled $H_2O$ | make up to 100 ml |

Dissolve tris in 80 ml $ddH_2O$, then adjust pH to 7.5. Add remaining water up to 100 ml. Autoclave at 121°C for 20 minutes.

### 1.2 EDTA

| | |
|---|---|
| EDTA | 186.12 g |
| Double distilled $H_2O$ | make up to 1000 ml |

Dissolve EDTA in 800 ml $ddH_2O$, then adjust pH to 8. Add remaining water up to 1000 ml. Autoclave at 121°C for 20 minutes.

### 1.3 TE buffer

| | |
|---|---|
| 1M Tris HCl | 10 ml |
| .5M EDTA | 20 ml |
| double distilled $H_2O$ | make up to 1000 ml |

### 1.4 CSOA selective agar

| | |
|---|---|
| Heart infustion broth | 25 g |
| agar | 15 g |
| double distilled $H_2O$ | make up to 998 ml |
| Colistin sulphate | 10 mg |
| Oxolinic acid | 5 mg |
| Ethanol | 1 ml |
| Double distilled $H_2O$ | 1 ml |

Dissolve first two ingredients and make up to 998 ml with double distilled water. Autoclave at 121°C for 20 minutes and cool to 50°C. Colistin sulphate and oxolinic acid purchased together as Streptococcus Selective Supplement (Oxoid, Australia). Dissolve supplement in 2 ml 1:1 ethanol:sterile distilled water. Add supplement to sterile HIA medium.

### 1.5 Phosphate buffered saline (PBS) × 10

| | |
|---|---|
| NaCl | 400 g |
| $KH_2PO_4$ | 10 g |
| $Na_2HPO_4$ | 57.5 g |
| double distilled $H_2O$ | make up to 5 L |

Adjust pH to 7.2.

**1.6 SM buffer**

| | |
|---|---|
| NaCl | 5.8 g |
| $MgSO_4.7H_2O$ | 2.0 g |
| 1M tris-HCl (pH 7.5) | 50 ml |
| 2% gelatin | 5 ml |
| double distilled $H_2O$ | make up to 1000 ml |

**1.7 2% gelatin**

| | |
|---|---|
| gelatin | 0.2 g |
| double distilled $H_2O$ | 10 ml |

Dissolve gelatin in double distilled water with heating and stirring. Autoclave at 121°C for 15 minutes.

**1.8 Ammonium sulphate solution (saturated)**

| | |
|---|---|
| Ammonium sulphate | 73 g |
| single distilled $H_2O$ | 100 ml |

Add ammonium sulphate to water and mix until completely dissolved.

**1.9 Destaining solution (10% acetic acid)**

| | |
|---|---|
| EtOH | 100 ml |
| Acetic acid | 100 ml |
| double distilled $H_2O$ | 800 ml |

**1.10 Protein elution buffer**

| | |
|---|---|
| Acetonitrile | 5 ml |
| Trifluoroacetic acid | 9 µl |
| double distilled $H_2O$ | make up to 10 ml |

**1.11 Sodium dodecyl sulphate (SDS) (20%)**

| | |
|---|---|
| Sodium dodecyl sulphate | 20 g |
| double distilled $H_2O$ | make up to 100 ml |

Dissolve SDS in 80 ml $ddH_2O$ with heating and stirring. Add remaining water up to 100 ml.

## APPENDIX 2

## POLYMERASE CHAIN REACTION PRIMERS AND REACTION CONDITIONS

| Primer name | Sequence (5'-3') |
|---|---|
| LOX-1 | AAGGGGAAATCGCAAGTGCC |
| LOX-2 | ATATCTGATTGGGCCGTCTAA |
| 27F | AGAGTTTGATCMTGGCTCAG |
| 1492R | TACGGYTACCTTGTTACGACTT |

**Reaction conditions for primer pair LOX-1/LOX-2:**

1. Denaturation 95°C 1 min
2. Denaturation 92°C 1 min
3. Annealing 55°C 1 min
4. Extension 72°C 2 min
5. (34 repetitions of steps 2-4)
6. Final extension 72°C 5 min

**Reaction conditions for primer pair 27F/1492R:**

1. Denaturation 94°C 5 min
2. Denaturation 94°C 1 min
3. Annealing 54°C 1 min
4. Extension 72°C 1.5 min
5. (29 repetitions of steps 2-4)
6. Final extension 72°C 7 min

# APPENDIX 3

# ENVIRONMENTAL SAMPLES

| Sample ID | Farm | Source | Material collected | Season Collected |
|---|---|---|---|---|
| EA1 | A | black water | water | summer |
| EA2 | A | pond | water | summer |
| EA3 | A | pond | sediment | summer |
| EA4 | A | pond | water and sediment | summer |
| EA5 | A | pond | water and sediment | summer |
| EA6 | A | pond | water and sediment | summer |
| EA7 | A | pond | water and sediment | summer |
| EA8 | A | pond | water and sediment | summer |
| EA9 | A | effluent | water and sediment | summer |
| EA10 | A | effluent | water and sediment | summer |
| EA11 | A | effluent | water and sediment | summer |
| EA12 | A | effluent | water and sediment | summer |
| EA13 | A | effluent | water and sediment | summer |
| EA14 | A | effluent | water and sediment | summer |
| EA15 | A | effluent | water and sediment | summer |
| EA16 | A | effluent | water and sediment | summer |
| EA17 | A | effluent | water and sediment | summer |
| EA18 | A | pond | water and sediment | summer |
| EA19 | A | pond | water and sediment | summer |
| EA20 | A | pond | water and sediment | summer |
| EA21 | A | pond | water and sediment | summer |
| EA22 | A | pond | water and sediment | summer |
| EA23 | A | pond | water and sediment | summer |
| EA24 | A | pond | water and sediment | summer |
| EB1 | B | settlement pond | water | summer* |
| EB2 | B | settlement pond | water and sediment | summer* |
| EB3 | B | settlement pond | water | summer* |
| EB4 | B | settlement pond | water and sediment | summer* |
| EB5 | B | settlement pond | water and sediment | summer* |
| EB6 | B | settlement pond | water and sediment | summer* |
| EB7 | B | raceway discharge | water | summer* |
| EB8 | B | nursery effluent | water and sediment | summer* |
| EB9 | B | raceway discharge | water and sediment | summer* |
| EB10 | B | raceway discharge | water and sediment | summer* |
| EB11 | B | raceway discharge | water and sediment | summer* |
| EB12 | B | raceway discharge | water and sediment | summer* |
| EB13 | B | raceway | water | summer* |
| EB14 | B | raceway | water and biofilm | summer* |
| EB15 | B | raceway | water | summer* |
| EB16 | B | raceway | water and biofilm | summer* |
| EB17 | B | raceway | water | summer* |
| EB18 | B | raceway | water and biofilm | summer* |
| EB21 | B | intake | water | summer* |
| EB22 | B | intake | water and sediment | summer* |
| EB23 | B | intake | water | summer* |
| EB24 | B | intake | water and sediment | summer* |
| EB25 | B | intake | water and sediment | summer* |

| | | | | |
|---|---|---|---|---|
| EB26 | B | intake | water and sediment | summer* |
| EB27 | B | intake | water and sediment | summer* |
| EB28 | B | intake | water | summer* |
| EB29 | B | effluent | water | summer* |
| EB30 | B | effluent | water and algae | summer* |
| EB31 | B | effluent | water and sediment | summer* |
| EB32 | B | effluent | water and sediment | summer* |
| EB33 | B | effluent | water and sediment | summer* |
| EB34 | B | effluent | water and sediment | summer* |
| EB35 | B | effluent | water | summer* |
| EB36 | B | effluent | water and sediment | summer* |
| EB37 | B | raceway | water | summer* |
| EB38 | B | raceway | water and sediment | summer* |
| EB39 | B | raceway | water | summer* |
| EB40 | B | raceway | water | summer* |
| EB41 | B | raceway | water and sediment | summer* |
| EB42 | B | raceway | water | summer* |
| EB43 | B | raceway | water and sediment | summer* |
| EB44 | B | raceway | water | summer* |
| EB45 | B | raceway | water and sediment | summer* |
| EB46 | B | raceway | water and sediment | summer* |
| EB47 | B | raceway | water | summer* |
| EB48 | B | raceway | water | summer* |
| EB49 | B | raceway | water | summer* |
| EB50 | B | raceway | water | summer* |
| EB51 | B | nursery effluent | water | summer* |
| EB52 | B | nursery effluent | water and sediment | summer* |
| EB53 | B | nursery effluent | water | summer* |
| EB54 | B | nursery effluent | water and sediment | summer* |
| EB55 | B | nursery effluent | water | summer* |
| EB56 | B | nursery effluent | water and sediment | summer* |
| EB57 | B | inlet | water | late autumn |
| EB58 | B | raceway | water and sediment | late autumn |
| EB59 | B | raceway | water | late autumn |
| EB60 | B | raceway | water and sediment | late autumn |
| EB61 | B | raceway | water | late autumn |
| EB62 | B | raceway | water and sediment | late autumn |
| EB63 | B | raceway | water | late autumn |
| EB64 | B | raceway | water and sediment | late autumn |
| EB65 | B | raceway | water | late autumn |
| EB66 | B | raceway | water and sediment | late autumn |
| EB67 | B | raceway | water | late autumn |
| EB68 | B | raceway | water and sediment | late autumn |
| EB69 | B | nursery effluent | water | late autumn |
| EB70 | B | nursery effluent | water and sediment | late autumn |
| EB71 | B | nursery effluent | water | late autumn |
| EB72 | B | nursery effluent | water and sediment | late autumn |
| EB73 | B | effluent | water | late autumn |
| EB74 | B | effluent | water and sediment | late autumn |
| EB75 | B | effluent | water | late autumn |
| EB76 | B | effluent | water and sediment | late autumn |
| EB77 | B | effluent | water | late autumn |
| EB78 | B | effluent | water and sediment | late autumn |
| EB79 | B | nursery effluent | water and sediment | late autumn |

| | | | | |
|---|---|---|---|---|
| EB80 | B | settlement pond | water and sediment | late autumn |
| EB81 | B | effluent | water and sediment | late autumn |
| EB82 | B | effluent | water and sediment | late autumn |
| EB83 | B | settlement pond | water and sediment | late autumn |
| EB84 | B | settlement pond | water and sediment | late autumn |
| EJCU1 | JCU | barramundi system | water | summer |
| EJCU2 | JCU | barramundi system outflow | water | summer |
| EC1 | C | pond | water and sediment | early summer |
| ED1 | D | dried pond | sediment | summer |
| EE1 | E | pond | water | late summer |
| EE2 | E | pond | water | late summer |
| EF1 | F | pond | water | late summer |
| EF2 | F | pond | water | late summer |
| EG1 | G | pond | water | late summer |
| EG2 | G | pond | water | late summer |
| EG3 | G | pond | water | late summer |
| EG4 | G | inlet | water | late summer |
| EG5 | G | effluent | water | late summer |
| EG6 | G | effluent | water | late summer |

*Farm B summer samples collected following heavy precipitation