THE UNIVERSITY OF QUEENSLAND

AUSTRALIA

Role of complement proteases in Streptococcus pathogenesis in fish

Nguyen, Chan D.H.

Master of Philosophy

A thesis submitted for the degree of Master of Philosophy at

The University of Queensland in 2015

School of Biological Sciences

Abstract

Streptococcal pathogens, particularly *Streptococcus iniae*, are emerging as a major cause of meningitis and septicaemia in a broad range of farmed and wild fish species, and are occasionally zoonotic. The major virulence factor in fish pathogenic streptococci is a highly variable antigenic polysaccharide capsule, which has resulted in serotype switching and reinfection of previously vaccinated fish. Therefore there is an urgent need to investigate more highly conserved protein-based critical virulence factors as targets for new vaccines. Complement proteases C5a peptidase and C3 protease of *S. iniae* were investigated in the present study to determine their role in virulence, as they are both secreted or surface associated and may represent potential targets for vaccination. My research shows that the C5a peptidase (scpI) and C3 protease (cppA) are inversely regulated with capsular polysaccharide. This coordinate regulation allows the capsule to be down-regulated to permit attachment to hydrophobic surfaces which may be essential for invasion of the Central Nervous System (CNS) and rapid onset of meningitis, whilst the proteases are up-regulated to protect the bacteria from complement-enhanced killing by neutrophils in barramundi whole blood assays. Knockout mutants deficient in each of the genes revealed reduced growth in whole blood, serum and antiserum. Moreover, these mutants revealed a role for ScpI and CppA in the interruption of the classical pathway since they were susceptible to the antiserum, which contained specific antibodies and complement proteins activating the classical pathway. Additional work to determine whether blood and serum resistance can be rescued by complementing the knockout mutants is required. To explore environmental factors in the host that may drive coordinate regulation of scpI and cppA with capsular polysaccharide synthesis protein E (cpsE) iron limitation was investigated. Iron stress appeared to be as one of the triggers for reduction the expression of capsule and coordinate increase in complement proteases expression. Future work should elucidate further environmental cues in the host that regulate expression of these genes, as it is likely to be complex and multifactorial. Together, these data implicate complement proteases in survival and dissemination in the host and therefore warrant further investigation as conserved protein targets for vaccines for farmed fish.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

I have clearly stated the contribution of others to my thesis as a whole, including statistical assistance, survey design, data analysis, significant technical procedures, professional editorial advice, and any other original research work used or reported in my thesis. The content of my thesis is the result of work I have carried out since the commencement of my research higher degree candidature and does not include a substantial part of work that has been submitted to qualify for the award of any other degree or diploma in any university or other tertiary institution. I have clearly stated which parts of my thesis, if any, have been submitted to qualify for another award.

I acknowledge that an electronic copy of my thesis must be lodged with the University Library and, subject to the policy and procedures of The University of Queensland, the thesis be made available for research and study in accordance with the Copyright Act 1968 unless a period of embargo has been approved by the Dean of the Graduate School.

I acknowledge that copyright of all material contained in my thesis resides with the copyright holder(s) of that material. Where appropriate I have obtained copyright permission from the copyright holder to reproduce material in this thesis.

Publications during candidature

Submitted paper
Chan N. Dieu, Andrew C. Barnes. Coordinate inverse regulation of putative complement-degrading proteases scpI and cppA with capsule in fish pathogenic *Streptococcus iniae*. Veterinary Microbiology (2015).

Publications included in this thesis

No publications included.

Contributions by others to the thesis

Associate Professor Andrew C. Barnes provided substantial intellectual support, supervision, funding, critical revision of entire thesis, and bioinformatics in Chapter II.

Mr. Michael Nefedov (School of Chemistry & Molecular Biosciences) provided assistance with Real-time PCR method and design.

Dr. Candice M. Health provided trouble shooting for making knock out mutants, and prepared the competent cells for electroporation described in Chapter III.

Dr. Gay Marsden provided general editing of thesis.

Statement of parts of the thesis submitted to qualify for the award of another degree

None.

Acknowledgements

Heading to Brisbane rather than Berlin was such a tough decision I had made ever, and it still confused me during the first month after arrival. It looked like I closed all the doors leading to human relating research to jump into a complete new field, the fish! Today, settling down and looking back the past two years I have spent here, that decision is worth in every single aspect. Though there were many experiences and obstacles during the time of doing my MPhil candidature, this was a splendid time for me to perceive challenges and also seduction from the marine biotechnology field. Therefore, it is the time and my pleasure to send a big thank to many people presenting in my MPhil adventure!

In the first honor place, I would like to send the greatest appreciation to my supervisor Andy Barnes, who has encouraged and supported me to go over the course. His intellectual support is definitely critical to make this thesis possible.

A very big hug to Gay Marsden, who has encouraged me jump into the pool! Thanks to her taking care of me in the first few months I've been here, and always checking whether I'm ok or not even she is working abroad.

Thanks to my entire lab mates who contributed this thesis with either intellectual or spirit supports. To Candice who introduced me to the world of knock out mutant, and kept encouraging me practicing my presentation skill. Thanks for her trouble shooting to not only knock out stuffs, but also others lab stuffs. To Kelly-Anne who always gives a satisfied answer to my every single question, she is also the one encouraged me purchase the first tennis racquet in my life! To Emma, Minami, Alex, and Jerome those helped me with the aquarium duties.

Lastly, I would like to thank to my family those live far away but always be in my heart. To my parents who have taken good care of me for 27 years since I was born, and keep doing it for another 27 years plus and plus. I know I am always their child even I'm getting mature now. I can't wait to see them in my graduation ceremony this July! Last but not least, to my fiancé Phong Phan, 'my cc', thanks for his love, patience, and waiting for me.

Keywords

Streptococcus iniae, complement, capsule, C3 protease, C5a peptidase, *ΔscpI*, *ΔcppA*.

Australian and New Zealand Standard Research Classifications (ANZSRC)

ANZSRC code: 060405, Gene Expression, 40%
ANZSRC code: 060501, Bacteriology, 40%
ANZSRC code: 060804, Animal Immunology, 20%

Fields of Research (FoR) Classification

FoR code: 0604, Genetics, 40%
FoR code: 0605, Microbiology, 40%
FoR code: 0608, Zoology, 20%

Table of contents

List of Figures

List of Tables

List of Abbreviations

aa	amino acid
ANOVA	Analysis of Variance
BBB	Blood brain barrier
BLAST	Basic Local Alignment Search Tool
CFS	Cerebral spinal fluid
CNS	Central Nervous System
CPS	Capsular polysaccharide
DMSO	Dimethyl sulfoxide
dNTP	Deoxynucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid

ELISA	Enzym-linked Immunosorbent Assay
FS	Fresh Serum
GAS	Group A Streptococci
GBS	Group B Streptococci
HIAS	Heat Inactivated Anti Serum
HINS	Heat Inactivated Normal Serum
IL	Interleukin
IM	Intramuscular
IP	Intraperitoneal
KO	Knock Out
MAC	Membrane attack complex
NCBI	National Center for Biotechnology Information
OD	Optical Density
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
RBC	Red Blood Cell
RPMI	Roswell Park Memorial Institute
RT	Room Temperature
SDS	Sodium sodecyl sulfate
THA	Todd-Hewitt Agar
THB	Todd-Hewitt Broth
TNF	Tumor Necrosis Factor
WT	Wild Type

1. Chapter I. General Introduction

Streptococcus species, particularly *Streptococcus iniae* are emerging as the most significant pathogens of warm water aquaculture fish species and cause serious economic loss to the industry worldwide (for review see Agnew and Barnes, 2007). It is also occasionally zoonotic, and is the most common cause of meningitis in neonates (for review see Baiano and Barnes, 2009). The disease symptoms presented in streptococcus infected mammals and aquatic species are very similar, namely septicemia and meningitis, in spite a reductive evolution of marine strains of Streptococci leading to extensive gene loss (Rosinski-Chupin et al., 2013). This provides an opportunity to explore conserved virulence factors amongst marine and terrestrial strains. C3 and C5a are two pivotal anaphylatoxins of the complement pathway activating a pro-inflammatory response of the innate immune system in both humans, and marine fish. Recent studies have indicated a pivotal role for these anaphylotoxins in the onset and development of meningitis (Gasque et al., 1998; Van Beek et al., 2000). Interestingly, the corruption of the complement system by the interference from a C5a peptidase and a C3-degrading protease has been demonstrated in *Streptococci* of group A, B, G in their pathogenesis in mammals (Cleary et al., 2004; Hostetter et al., 2004; Ji et al., 1996). Genes encoding these proteins are retained in fish pathogenic streptococci, including *Streptococcus iniae* (Baiano and Barnes, 2009; Locke et al., 2008; Zhang et al., 2014). Could manipulation of the complement system play a role in the meningeal pathology that results from these infections? I aim to understand how marine streptococcal pathogens manipulate the complement system and subsequently determine whether there is a role for complement manipulation in the development of meningitis during fish infection. This research will help to identify key pathways in pathogenesis and identify putative surface targets for future fish vaccines for aquaculture.

1.1 Host defences to infection of the brain and central nervous system

Meningitis is a serious condition that is characteristic of streptococcal infections in fish, in the case of *S. iniae*, and in humans. It is the inflammation of the meninges, which protect the brain and spinal cord. The cerebrospinal fluid contained in the meninges is a source of bacteria that may infect and cause the inflammation. In order to understand how *S. iniae*

approaches, invades and causes meningitis in fish, an appreciation of how the host's central nervous system defends itself against pathogens is required, including the concept of the blood-brain barrier and how it interacts with the innate immune system. Moreover current understanding of strategies for adherence and invasion by streptococcal pathogens will be examined.

1.1.1 Blood brain barrier and its role in innate immunity

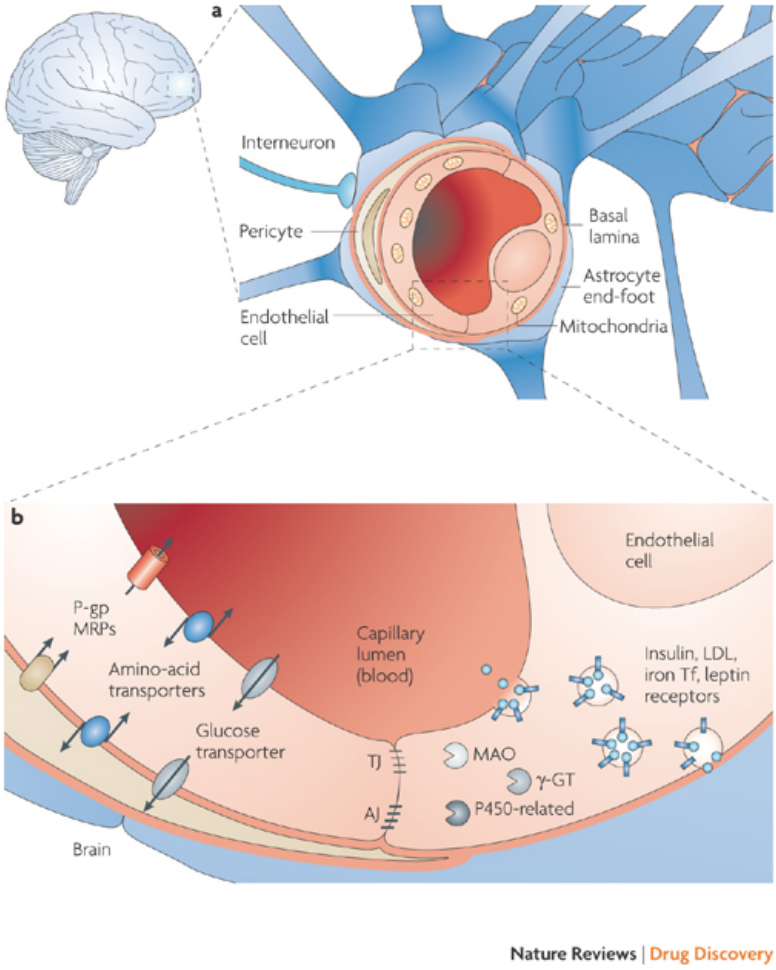

Figure 1. The blood-brain barrier (BBB) model. BBB is constructed from multilayers in which (a) adjacent microvascular endothelial cells are the main component and (b) connected via tight junction (TJ) and adherent junction (AJ). (photo by Cecchelli et al., 2007)

The blood-brain barrier (BBB) is constructed from multilayers in which adjacent microvascular endothelial cells are the main component (Brightman, 1977) (Figure 1). These endothelial cells are connected by adherent junctions and tight junctions at their intercellular contacts (Schulze and Firth, 1993; Wolburg and Lippoldt, 2002). Based on

these interactions between junctions, the endothelial cells maintain their functions of cell polarity, signaling, cell stabilization and thereby BBB integrity (Braga, 2002; Matter and Balda, 2003; Wheelock and Johnson, 2003) which is vital to preventing the invasion of pathogens. Adjacent to endothelial cells are pericytes (Johanson, 1980). These endothelial cells and pericytes are surrounded by a basement membrane (Johanson, 1980) which is in turn surrounded by the foot of the astrocyte and neuron endings (Cohen et al., 1995; Johanson, 1980; Paspalas and Papadopoulos, 1996). The communications between endothelial cells, astrocytes, pericytes are unique cell-cell interactions because of the very short distance between these cells (only 20 nm) (Pardridge, 2003; Paulson and Newman, 1987).

The BBB has sparse pinocytic activity which restricts large molecules and pathogens from entering cells and limits the amount of transcellular flux. It is the last barrier to bacteria attempting to enter the homeostatic environment of the Central Nervous System (CNS). The multiplication of pathogens within the subarachnoid space is rapid in the absence of inhibition by large molecules of the host immune system such as complement and immunoglobulins (Ransohoff et al., 2003). Due to this deficiency in host humoral defense response within the CNS, migration of leukocytes through BBB compensates for this weakness. Indeed, various cells within the BBB and CNS produce the pro-inflammatory cytokines tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6) (Tauber and Moser, 1999; van Furth et al., 1996) involved in leukocyte recruitment and activation. In summary, the BBB plays a role in the immune alert system prior to invasion of the CNS, but over-activation may occur with continuous insult from a pathogen leading to a loss of BBB integrity (van Sorge and Doran, 2012).

1.1.2 Complement pathways – innate immune system

1.1.2.1 Complement pathways

The complement system is an ancient innate immune pathway that has existed for over 350 million years and has been found in organisms as primitive as the horseshoe crab (*Carcinoscorpius rotundicauda*) (Zhu et al., 2005) and scleractinian corals (Kvennefors et al., 2010). Although the complement system is not adaptable and does not change over the lifespan of the host, it can be recruited by the adaptive immune system (Janeway,

2001). Employing over thirty plasma proteins, the complement system is a very complex network consisting of a cascade of proteolytic events providing immunity against a wide variety of pathogens. The major functions of the complement system are opsonizing invaders for immune and inflammatory cells, stimulating the immune system and finally contributing to the destruction of pathogens as the membrane attack complexes (MAC) are formed at the cell surface (Ha□nsch et al., 1998; Liszewski et al., 1996). In addition, the complement system plays an important role in regulation of humoral immunity (Molina et al., 1996), modification of T cells (Kaya et al., 2001), control of the development of antibody repertoire (Fleming et al., 2002), and regulation of self-reactive B lymphocytes that produce anti-nuclear and anti-dsDNA antibodies (Carroll, 2000; Prodeus et al., 1998). It is activated via three pathways, classical, lectin, and alternative pathway (Figure 2).

The classical pathway is activated by the response of the C1 complex to the interaction of IgM or IgG1 and IgG3 with antigen in humans (Mak and Saunders, 2007). The lectin pathway is activated by mannose-binding lectins (MBL) (Reid and Turner, 1994), Ficolins (Matsushita et al., 2000), and cytokeratin, is present on ischemic endothelial cells (Collard et al., 1998) which can recognize the sugar residues anchoring on the pathogen surface. The alternative pathway does not require the specific interaction of protein-protein or protein-sugar for activation. It has a wide range of activators including bacterial polysaccharides and lipopolysaccharides, viral infected cells, yeast cell walls, IgA containing immune complexes, inulin, cobra venom factor, and especially, transformation of C3 into C3(H_2O), subsequently C3b (Yancey and Lazarova, 2008).

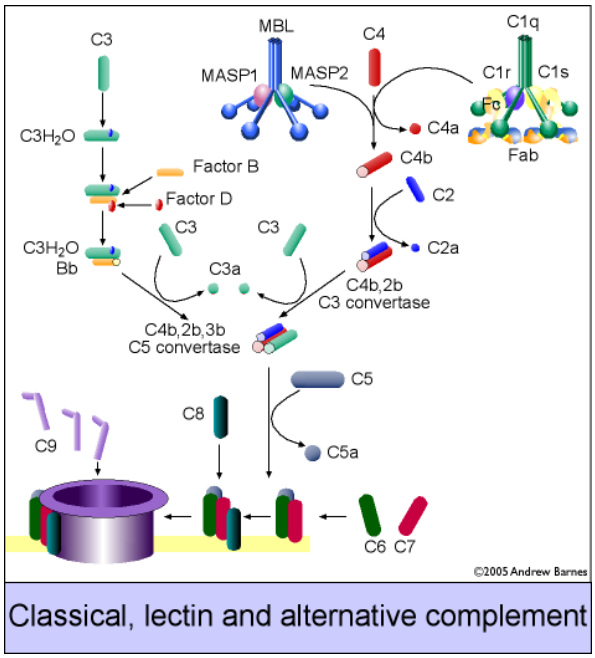

Figure 2. The classical, lectin, and alternative complement pathways

These pathways are differentiated at the initiation and follow three distinct reaction cascades, but they share most components and generate the same variants of the protease C3-convertase and C5-convertase (Ha□nsch et al., 1998; Huber-Lang et al., 2006). All three pathways share similar downstream formation of membrane attack complexes (MAC). The MAC is a transmembrane channel that can disrupt the phospholipid bilayer of the pathogen cell surface leading to the cell lysis and death. Whether cleaved by classical, lectin or alternative C5-convertase, C5 is fragmented identically resulting in two molecules C5a and C5b. After interacting with C6 to form the C5b6 complex, other terminal complement components (i.e. C7, C8) are attracted resulting in the formation of complement complexes that are capable of attaching and associating deep within the lipid bilayer and thereby disrupting the plasma membrane (Lachmann and Thompson, 1970; Thompson and Lachmann, 1970). Finally, C9, which is amphiphilic and capable of polymerization, binds to C5b678 to form a transmembrane tubular complex poly-C9 (Podack and Tschopp, 1982) that eventually lyses the target cell.

1.1.2.2 Complement system in fish

The complement system in aquatic animals has similar properties to their mammalian counterparts (Boshra et al., 2006; Holland and Lambris, 2002). Cytolysis and opsonisation

for later phagocytosis by the fish complement system have been confirmed in a range of fish species (Byon et al., 2006; Gonzalez et al., 2007; Jenkins and Ourth, 1993; Lammens et al., 2000; Matsuyama et al., 1992; Moritomo et al., 1988; Overturf and LaPatra, 2006; Saeij et al., 2006; Sakai, 1984; Sigh et al., 2004). The fry of trout and masu salmon, which do not possess complement activity at this stage of development, are more susceptible to infectious haematopoietic necrosis virus infectious pancreatic necrosis virus than their Atlantic salmon fry counterpart which already possess complement activity in serum (Yano, 1997). In rainbow trout, the complement components present in the skin and serum can kill the parasites *Gyrodactylus derjavini*, *Gyrodactylus salaris*, *Ichthyophthirius multifiliis*, *Cyptobia salmositica* through both classical and alternative complement pathways (Buchmann, 1998; Buchmann et al., 2001; Harris et al., 1998; Woo, 1996). Interestingly, the fish complement proteins, which are structurally similar to those of mammals, appear to possess many isoforms (e.g. C3 and factor B) which may be transcribed from more than one gene (Holland and Lambris, 2002). Indeed, the genes coding for C3 in fish are polymorphic and may lead to multiple mechanisms of activation (Jensen and Koch, 1991; Nonaka et al., 1985).

C3- and C5-convertase are universal complexes formed by three pathways, and they are essential for the formation of key components C3a and C5a thus are essential to progression of the lytic complement pathway, initiation of the pro-inflammatory response and activation of migrating leukocytes.

1.1.2.3 C3a and C5a Anaphylatoxins

The proteolytic reactions of C3, C4, and C5 result in lower molecular-weight fragments C3a, C4a, and C5a that are anaphylatoxins based on their pro-inflammatory action, promoting the release of vasoactive mediators from mast cells and basophils (Gorski et al., 1979; Yancey, 1988). They share a sequence homology of up to ≈35%, a carboxy-terminal arginine and the relative order of activity strength descends from C5a to C3a, then C4a (Barnum et al., 2002; Dahinden et al., 1991; Elsner et al., 1994; Hartmann et al., 1997; Norgauer et al., 1993; Settmacher et al., 1999; Van Epps et al., 1993).

C5a weighs 12-14.5 kDa and is composed of 74-amino acids, in which Asn64 has N-linked carbohydrate that is likely control the activity of C5a *in vivo* (Monk et al., 2007). In terms of

activity, C5a is 200 times more potent than C3a (Yancey and Lazarova, 2008) and its effects are pleiotropic including induction of smooth muscle contraction (Gasque et al., 1998; Haviland et al., 1995; Zwirner et al., 1999), stimulation of histamine from mast cells, chemoattraction of neutrophils, monocytes, macrophages (Becker, 1972; Snyderman et al., 1970; Snyderman et al., 1975), eosinophils (Kay et al., 1973), basophils (Hook et al., 1975), liver Kuppfer cells (Laskin and Pilaro, 1986), microglia in the CNS (Yao et al., 1990), and also B and T lymphocytes (El-Naggar et al., 1980). In addition, C5a also participates in activating oxidative burst in neutrophils, and promotes phagocytosis (Goldstein and Weissmann, 1974; Guo and Ward, 2005; Marder et al., 1985; Sacks et al., 1978). The over-expression of C5a is found in inflammatory disorders implying under-regulation in these diseases (Monk et al., 2007).

C3 is a 185 kDa protein and is the dominant complement protein in terms of relative quantity (Haas and van Strijp, 2007). C3 consists of two chains, α- and β-chain, which are cleaved at the site between Arginine-77 and Serine-78 on the α-chain by C3-convertase of any of the classical, lectin, or alternative pathways, resulting in fragments C3a and C3b (Beutler, 2004). C3b continuously combines with other complement components to form the proteolytic complexes. C3a, which is a 77-amino acid fragment, is a pro-inflammatory mediator participating in cellular and physiological events including contraction of smooth muscle, activation of leukocytes, eosinophils, and promotion of vascular permeability (Gerard and Gerard, 2002; Hawlisch et al., 2004). C3a causes a broad-ranging pro-inflammatory effect and C3a receptors are found on activated lymphocytes and endothelial cells during the inflammation of the brain (Fischer and Hugli, 1997; Francis et al., 2003; Gasque et al., 1998; Monsinjon et al., 2001).

In summary, the two anaphylatoxins C3a and C5a are the most potent pro-inflammatory mediators and are integral to all three of the complement pathways thus constituting efficient evolutionary targets for interference by proteases of pathogenic bacteria.

1.2 *Streptococcus iniae* virulence factors and the onset of meningitis

When *Streptococcus iniae* invades the host and enters the bloodstream it uses the polysaccharide capsule as a shield against the host defense system, particularly phagocytosis and complement (Doran et al., 2003; Kim et al., 1992; Nizet et al., 1997; St

Geme and Falkow, 1991; Unkmeir et al., 2002). However, meningeal pathogens depress capsular expression prior to adhesion to host cells that may be required during attack on the CNS (Hammerschmidt et al., 1996; Ring et al., 1998; St Geme and Cutter, 1996; St Geme and Falkow, 1991). The reduction in host-cell adhesion when capsule is expressed is a result of decreased exposure of critical surface proteins and/or through electrostatic repulsion leading to failure of binding to host cells (Hammerschmidt et al., 1996; Ring et al., 1998). However, in the case of insufficient capsular cover, the pathogen is at high risk of clearance by the host's innate immune system, particularly the complement system, unless it initiates another protecting system.

Some bacterial pathogens have evolved mechanisms to resist host complement that include avoiding, exploiting or disrupting the complement system (Kuo et al., 2008; Terao et al., 2008; Tsao et al., 2006). Invaders may (*i*) utilize host complement regulators (e.g. factor H and factor H-like protein (Zipfel et al., 2002)), (*ii*) employ bacterial complement inhibitors (e.g. CD59-like protein from *Borrelia burgdorferi* (Pausa et al., 2003)),and (*iii*) degrade complement components by bacterial proteases (Rooijakkers and van Strijp, 2007). The degradation and/or modulation of the functional activity of complement proteins by bacterial proteases are considered essential and almost exclusive invasion strategy of many bacterial pathogens. It involves directly degrading the substrates or incorporating them with host proteases to attenuate them (Michal and Jan, 2012).

Bacterial proteases are employed by mammalian pathogens however it is unknown whether marine counterparts employ the same systems. The disease symptoms caused by Streptococcal species are quite similar despite evolutionary gene loss which presents an opportunity to explore conserved virulence factors amongst the reduced genomes and their human and mammalian counterparts.

1.2.1 *Streptococcus iniae* Pathophysiology

The clinical symptoms of *S. iniae* can vary between host species. In humans, symptoms include bacteraemic cellulitis in organs or joints, endocarditis, meningitis, osteomyelitis, and septic arthritis (Facklam et al., 2005; Lau et al., 2003; Weinstein et al., 1997). In dolphins multifocal subcutaneous abscesses are the only recorded symptom to date (Bonar and Wagner, 2003; Pier and Madin, 1976; Pier et al., 1978). In tilapia (*Oreochromis*

aura, *Oreochromis nilotica*), trout (*Oncorhynchus mykiss*), common carp (*Cyprinus carpus*), grey mullet (*Mugus cephalus*), yellowtail (*Seriola quinqueradiata*), Coho salmon (*Oncorhynchus kisutch*) and barramundi (*Lates calcarifer*), clinical signs are the loss of orientation, melanosis, tachypnea, anorexia, emaciation, corneal opacity, mild peritonitis, lethargy, petechiae around the anal zone, gill rot, dorsal rigidity, vertebral deformity, scattered haemorrhages in the body cavity, osteomyelitis and sudden death have also been reported in acutely infected fish (Eldar et al., 1994; Eldar et al., 1995; Eldar and Ghittino, 1999; Eldar et al., 1997; Kaige et al., 1984).

1.2.2 *Streptococcus iniae* Virulence factors

Virulence factors that present on the pathogen surface or are secreted are critical in the promotion of adhesion, invasion, and resistance to the host immune system. They are also potential targets for vaccination due to their exposure to the adaptive immune system during infection. In *S. iniae*, the predominant virulence factor is its capsule, which has been found in almost all virulent isolates, and confers resistance to phagocytosis by fish macrophages (Locke et al., 2007). This capsular polysaccharide can cover surface antigens, prevent opsonisation by complement C3 and macrophage clearance (Barnes et al., 2003; Buchanan et al., 2005; Miller and Neely, 2005).

The secreted enzymes playing roles in *S. iniae* pathogenesis consists of IL-8 protease, C5a peptidase, streptokinase, and superantigens (Nobbs et al., 2009). IL-8 protease is responsible for cleavage IL-8 produced as a pro-inflammatory cytokine by the host innate defense system, resulting in increased bacteria survival and proliferation (Zinkernagel et al., 2008). Interfering with host complement pathway, C5a peptidase is postulated to hydrolyse the strong anaphylatoxin C5a (Locke et al., 2008; Tamura et al., 2004). In *S. iniae*, C5a peptidase is 123 kDa, has similar structural features to those of Group A Streptococci (GAS), and possesses classical Gram-positive anchor motif LPXTG (Locke et al., 2008). However, the contribution of this protein to *S. iniae* virulence is uncertain as allelic exchange of its encoding gene, *scpI* did not result in substantial attenuation of virulence in fish (Locke et al., 2008).

1.2.3 *Streptococcus iniae* Pathogenesis leading to Meningitis

The general bacterial pathogenesis leading to meningitis is a multistep infection process including the colonization followed by entering the CNS; then bacterial replication occurs in the subarachnoid space and ventricular system. This leads to a host inflammatory response which results in damage of the neural system (Leib and Täuber, 1999).

1.2.3.1 Entry of infection and colonization

Route of infection by *S. iniae* was explored by Evans *et al.* (2000) in hybrid striped bass (*Morone chrysops* x *Morone saxatilis*) and tilapia (*Oreochromis niloticus*) (Evans et al., 2000), suggesting common route of infection via nares. Barramundi (*Lates calcarifer*) can be infected with *S. iniae* via many routes including oral, bath exposure, intra-peritoneal injection, olfactory route, contact with or cannibalization of infected fish (Bromage and Owens, 2002; Shoemaker et al., 2000). In 2003, Zlotkin *et al.* suggested that the pathogenesis of *S. iniae* may be initiated by colonization of external or gastrointestinal tissues, from which it starts spreading by entering the blood stream (Zlotkin et al., 2003). Once the meningitis pathogens translocate to the sub-epithelial blood vessels, their colonization is enhanced depending on the surface components of that pathogen including fimbriae, capsule, and exonuclease (Leib and Täuber, 1999).

1.2.3.2 Survival within bloodstream

Once inside the blood vessels and intra- or inter-cellular space, pathogens must battle against the hosts defense mechanisms (antibodies, complement pathways and phagocytosis) (de Vries et al., 1996). The first defense that pathogens must face is the non-specific alternative and lectin complement pathways of the host. Once complement is activated, complement C3 is cleaved resulting in the binding of C3b on bacterial surface followed by a cascade of complement component cleavage and binding as well as opsonisation (enhancing ‘visibility’) for phagocytosis. Capsular polysaccharide is critical for avoiding phagocytosis and for resistance to complement mediated lysis during this phase of rapid expansion (Eyngor et al., 2010; Lowe et al., 2007; Martins et al., 2010).

1.2.3.3 Breaching Blood Brain Barrier and penetration of the Cerebral Spinal Fluid

The movement of blood-borne pathogens through the BBB starts with their attachment to the brain microvascular endothelial cells. Meningeal pathogens are aided by receptors

located within the endothelium of the choroid plexus and cerebral capillaries as well as by low blood flow in the choroid plexus (Mairey et al., 2006). From this state, the pathogen can penetrate (*i.e.* transcellularly) the endothelial cell (Das et al., 2001; Greiffenberg et al., 1998; Jain et al., 2006; Nikulin et al., 2006; Nizet et al., 1997; Prasadarao et al., 1999; Ring et al., 1998; Stins et al., 2001; van Sorge et al., 2011) or move aside (*i.e.* paracellularly) the endothelial cells via junction route (van Sorge and Doran, 2012) to enter the cerebral spinal fluid (CSF) compartment. Once the pathogens enter the endothelial cells, inflammation will be stimulated by cytokine activation which increases the amount of receptors capable of binding with phosphorylcholine; this creates an advantage for some bacterial pathogens to further their invasion (Banerjee et al., 2010; Cundell et al., 1995; Ring et al., 1998; Zhang et al., 2002).

The successful adherence and invasion of meningeal pathogens with ineffective capsules (Doran et al., 2003; Nizet et al., 1997; Unkmeir et al., 2002) shows that pathogens have an alternative strategy which enables them to remain virulent and protect them from host phagocytosis, but also that capsule is not required, or may even be an impediment to host cell adhesion. This means, at least in terms of capsular polysaccharide, there is a trade-off between resistance to innate immune defences and the adhesion that must occur in order to initiate crossing of the BBB.

1.2.4 Putative complement proteases in *Streptococcus iniae*

1.2.4.1 C5a peptidase

C5a peptidase Scp (Streptococcal C5a peptidase) is a branch of the subtilisin family of serine proteases, which can cleave C5a near its N-terminus, i.e. between His-67 and Lys-68 within the binding site for polymorphonuclear leukocytes, resulting in the recognition region of C5a with its receptor is removed (Wexler et al., 1985). The disruption of C5a results in the slowdown of influx of polymorphonuclear neutrophil leukocytes through failure of chemotactic signaling and neutrophil attraction to the infection site (Cleary et al., 1992). Interestingly, unlike other serine proteases, Scp only acts on C5a, not C5 or other proteins (Ji et al., 1996). Based on the cleavage of C5a at an inaccessible site His-Lys, and the observation that C5 is resistant to Scp, it strongly indicates that Scp is highly specific for C5a (Cleary et al., 1992).

Group A, B, C, G Streptococci, the β-hemolytic species, express a cell wall-anchored protein C5a peptidase Scp (Hill et al., 1988). Both Group A and B Streptococci possess the mechanism to escape from phagocytosis. The clearance of GAS at the infection site is delayed when Scp is expressed on its surface (Hung et al., 2012). Additionally, ScpB acts as an adhesin to Fibronectin type III and enhances invasion of epithelial cells (Beckmann et al., 2002). Interestingly, anti-ScpB antibody opsonizes Group B Streptococci (GBS) across serotypes, resulting in increased killing rate and macrophage oxidative burst (Cheng et al., 2001). It is possible that a novel vaccine for GBS will be made from ScpB, and similarly with ScpA in GAS. Scp is highly conserved among species, and the similarity of Scp proteins produced in GAS and GBS is up to 98% (Chmouryguina et al., 1997; Chmouryguina et al., 1996). For consistency, Scp is generally known as ScpA in GAS, ScpB in GBS, and ScpI in *S. iniae,* which is not type-able by the Lancefield system.

In GAS, ScpA consists of 1167 amino residues and is 128,814 Da. The signal sequence comprises the first 31 amino acid, including hydrophilic residues as a basement, hydrophobic as a core, and a cleavage site (Chen and Cleary, 1990; von Heijne, 1983). The next 68 amino acids, the propeptide, are also removed in the process of maturation of the Scp protein (Anderson et al., 2002). The highly conserved active site of ScpA is formed by Asp130, His193 and Ser512 that locate in the NH_2-terminal (Chen and Cleary, 1990; Siezen et al., 1991). In GBS, ScpB is also translated in the same manner as GAS (Chmouryguina et al., 1996) in that cleavage occurs between Ala31 and Arg32. Also, the active site triad Asp130, His193 and Ser509 is conserved (Chen and Cleary, 1990; Siezen et al., 1991). Located next to the carboxy terminus is a region of glycine and proline, which is an anchor-region for the peptidoglycan cell wall (Fischetti, 1989). This cell wall attachment domain is deduced as 20-residue hydrophilic sequence, a hydrophobic core of 17 residues which stretch from the glutamic acid residue at position 1029, and a COOH-terminus (Chen and Cleary, 1990; Janulczyk and Rasmussen, 2001; Schneewind et al., 1995; Uhlen et al., 1984) (Figure 3).

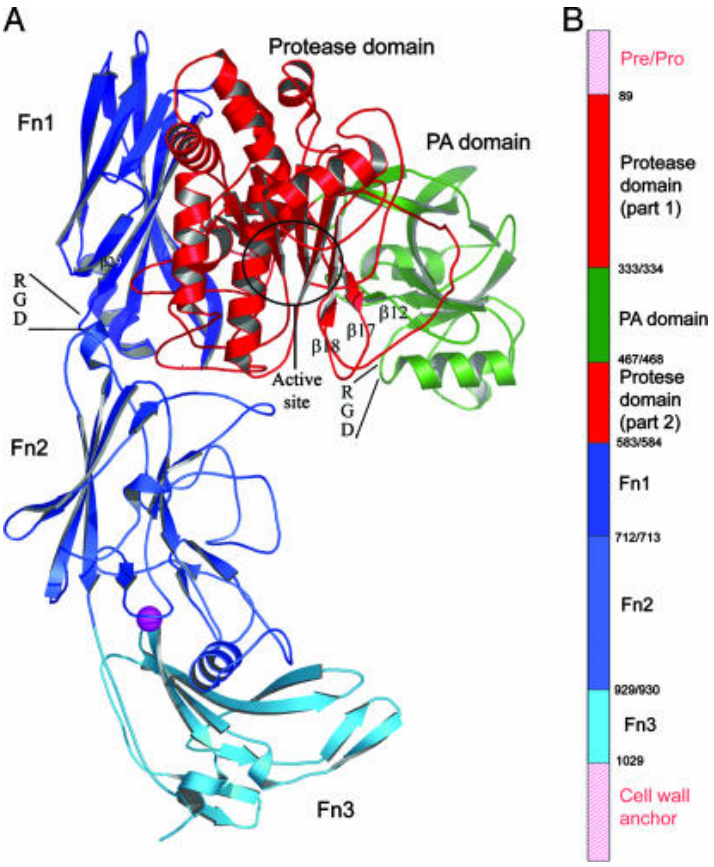

Figure 3. (A) A ribbon drawing of ScpB monomer. Proteases domain is red which contains the active site region (circle), protease-associated domain is green, and the remainder comprises three Fibronectin domains. (B) The schematic arrangement of ScpB in linear form. The colors representing domains are as in (A). The pink regions, which are the pro-domain and cell wall anchor, are not present in mature form of ScpB. (Brown et al., 2005)

The C5a protease-like protein ScpI in *S. iniae* also possesses the serine protease triad active sites Asp130, His193, and Ser512, however these amino acids are distributed in a slightly different manner to Asp114, His181 and Ser501 (Locke et al., 2008). This may suggest that the proteolysis function of this Scp-like protease plays a minor role in the virulence of *S. iniae* (Locke et al., 2008).

In 2008, Locke *et al.* identified a candidate *Scp*-like gene in *S. iniae* strain K288 named *ScpI* with sequence similar (37% identity and 55% positive (Locke et al., 2008)) to GAS Manfredo M5 strain (Holden et al., 2007) and GBS *S. agalactiae* strain A909 (Tettelin et al., 2005). *scpI* is a gene of 3369 bp at position 952620-955988 distally from *simA*, which encodes for M-like protein (Locke et al., 2008). The position in *S. iniae* is more similar to that of Group C and G Streptococcus rather than to GAS (Geyer and Schmidt, 2000).

Upstream of *ScpI* is a sugar ABC transporter gene *satA* which is 957 bp and identical to that of GAS (Ferretti et al., 2001). Adjacent downstream of *scpI* is 237 bp, a divergently transcribed transposase *tnpA* (Locke et al., 2008). Proximally located, upstream of *tnpA,* is 498 bp phosphoglucomutase gene *pgmA* which is involved in polysaccharide expression in *S. iniae* and contributes to virulence in fish (Buchanan et al., 2005). In the latest published *S. iniae* genome on GenBank in October 2013 (gene ID 16208933), the *scpI* of *S. iniae* strain F1 has slightly different neighbouring genes. Starting from the left, the first is a gene encoding for ABC transporter-ATP-binding protein, next is a branched-chain amino acid ABC transporter-permease protein encoding gene, followed by a *satA* gene, the *scpI* gene and finally two divergently transcribed genes of PgmA and membrane protein.

The ScpI-like protein likely possesses conserved domains with GAS and GBS, consisting of two peptidase regions flanking the protease-associated domain, and Fibronectin III domains (Figure 4).

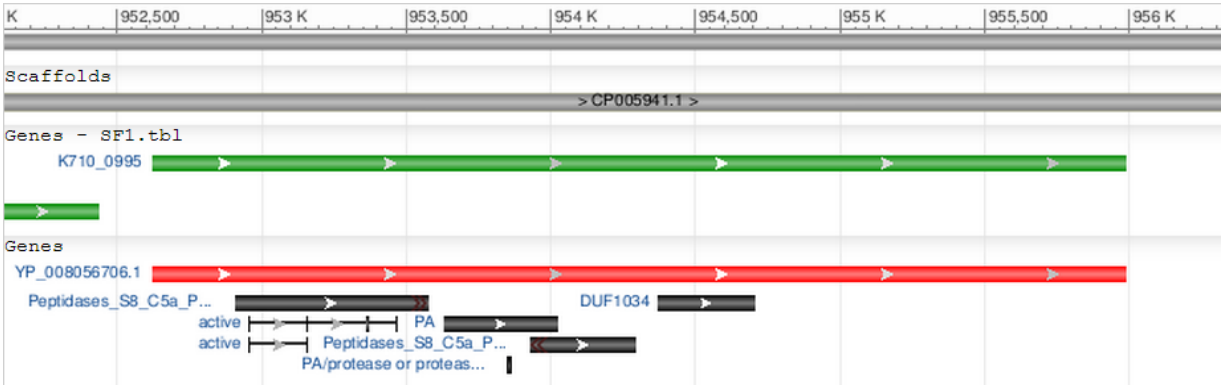

Figure 4. scpI encoding gene of S. iniae SF1 (GenBank ID 16208933). The black regions represent for protease part 1, protease-associated, protease part 2, and fibronectin domains, respectively.

1.2.4.2 The putative C3-degrading protease

The CppA protease is the surface peptidase that was first described in *Streptococcus pneumoniae* being capable of degrading the human complement protein C3 by Hostetter *et al* in 2004 (Hostetter et al., 2004). The molecular weight of this proteases isolated from the wild type *Streptococcus pneumoniae* ranges from about 24 kDa to 34 kDa determined by 10% SDS-PAGE (Hostetter et al., 2004) and the protein residue of cppA protein among Streptococci is variable e.g. 241 amino acid (aa) in *S. pneumoniae* (WP_001102220.1)

(Tettelin et al., 2001), 250 aa in *S. dysgalactiae subsp. equisimilis* (YP_002997602.1) (Shimomura et al., 2011), 250 aa in *S. pyogenes* (NP_269848.1)(Ferretti et al., 2001), 251 aa in *S. agalactiae* (WP_000171842.1) (Tettelin et al., 2005), 255 aa in *S.salivarius* (WP_014632660.1) (Guedon et al., 2011). Human C3 complement protein is completely preferentially degraded β-chain than α-chain that without produced the defined fragments, which can be observed on SDS-PAGE (Angel et al., 1994); however the CppA isolated from *S.pneumoniae* preferentially cleave the α-chain than the β-chain (Hostetter et al., 2004). This protease is highly conserved among the *Streptococcus equinus*, *Streptococcus bovis* and considered as a virulence factor together with others i.e. adhesion factors, immunoreactive antigens, superantigens, toxins, DNases as well as an factor involving in the capsule expression regulation (Kadioglu et al., 2002; Papadimitriou et al., 2014b). In 1994, the C3-degrading activity was demonstrated on the capsulated and uncapsulated *S. pneumoniae* that the degradation occurred independently with the capsule expression (Angel et al., 1994). This previous study also demonstrated the degrading activity of substantial proportion of soluble C3 occurred within 30 min in the exponential growth phase, and this degradation occurred before the opsonizing because the cleaved fragment generated by proteolytic degradation of opsonized C3b was not obtained (Angel et al., 1994). In *Streptococcus pneumoniae*, isogenic C3-degrading protease mutants cause less mortality and tissue damage than the wild type (Hostetter et al., 2004). In terms of complement pathway corruption, CppA can cleave the C3 component and prevent the formation of the C5 convertase complex. The cleavage of C3 leads to failure of opsonizing bacteria by C3b, which prevents the formation of extreme pro-inflammatory mediator C5a, the assembly of MAC and ultimately phagocytosis by neutrophils. In GBS, the decrease of virulence in the invasion was demonstrated that being related to the decrease of C5a peptidase and C3-degrading protease (Sharma et al., 2013).

In the genomic sequence analysis of *S. iniae*, Locke *et. al.* (2008) suggested that a putative C3 protease encoding gene may inactivate the process upstream of C5a peptidase (Locke et al., 2008). The putative C3-degrading protease was published in the complete genome sequence of *S. iniae* SF1 isolated from fish by Zhang et al (GenBank ID AGM99589.1) (Zhang et al., 2014). However in the two later complete sequences of *S.iniae* from Tilapia i.e. strain ISNO and ISET0901, this protein was listed as peptidase

(AHY18370.1 and AHY16506.1) (Pridgeon et al., 2014a, b) although it has 99-100% identity to C3-degrading protease of Zhang *et al.,* (2014) by BLAST, which warrants further investigation. The protease coding gene *cppA* was also found in other Streptococci such as *S. dysgalactiae* subsp. *equisimilis* that is closely related to *S. iniae* (Shimomura et al., 2011). Among the virulence factors that may enhance pathogenesis, *cppA* appears to be conserved in Streptococci despite of the various positions of *cppA* in their genome. In addition, the multi-alignment of *cppA* among *S. agalactiae, S. mutants, S. pneumoniae, S. pyogenes, and S. sanguinis* indicates the number of variable sites is 579 (76.89%) out of 753 bp aligned.

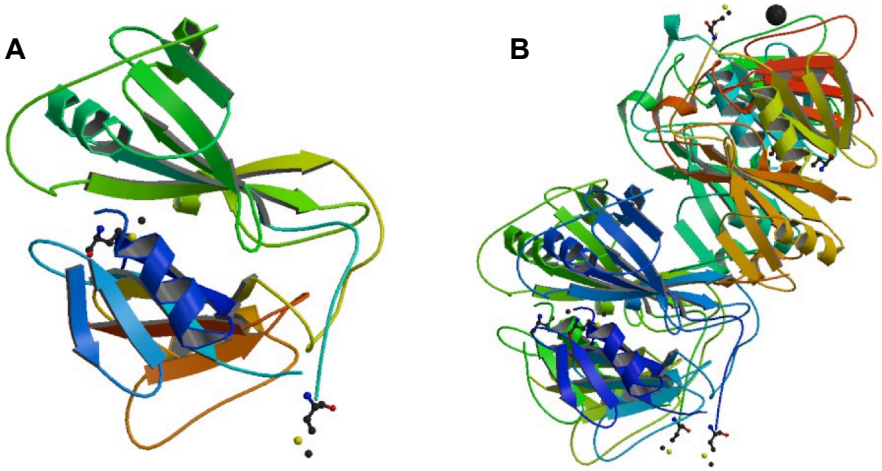

Figure 5. C3-degrading protease CppA, the ribbon model of a proteinase enzyme from the TIGR4 strain of Streptococcus pneumonia. (A) Monomeric unit. (B) Asymmetric unit, this model consists of four protein chains. (Nocek B. et al, 2008)

The putative C3-degrading protease encoding gene *cppA* contains four open reading frames in which the third ORF is interrupted by the homologous recombination impairing the C3 degradation i.e. the translated protein from ORF3 does not share the substantial homology to the others (Hostetter et al., 2004). Also, the CppA encoding gene is translated into N- and C-terminal chains that are clearly specified in *S. pyogenes* (Figure 6), however in *S. iniae* those regions are currently noticed as GLo_EDI-BRP_like domains, which belong to the superfamily found in the variety of structurally related metalloproteins (Figure 7). In term of coordinate genes regulation, C3-degrading protease encoding gene has been demonstrated that its expression has relation with capsule in *Streptococcus bovis*

and *Streptococcus equinus* (Papadimitriou et al., 2014a).

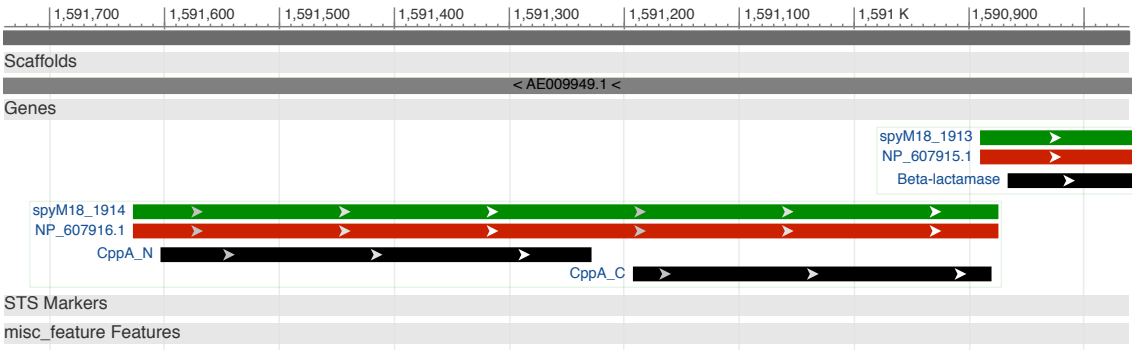

Figure 6. cppA encoding gene of S. pyogenes (GenBank ID NC_004070.1), in which N- and C-terminal chains have been identified.

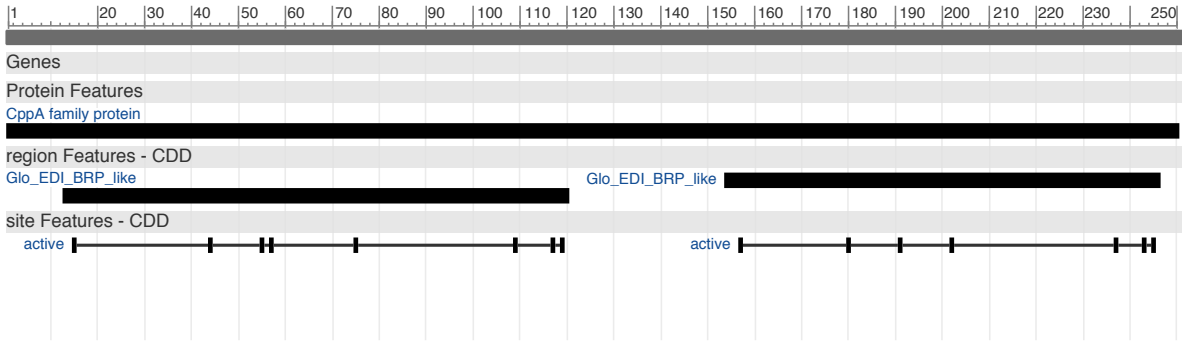

Figure 7. cppA encoding gene of S. iniae (GenBank ID NC_021314.1), in which the active regions are mentioned as the GLo_EDI_BRP_like regions.

1.3 The effect of growth phase on gene expression

During growth, bacteria themselves change the conditions of their environment as a direct consequence of the cost of their increasing density through production of toxic metabolites and exhaustion of vital micro and macronutrients, which explains why they have different growth phases, i.e. growth rates, during their life cycle in closed culture systems. Recent studies have demonstrated that this phase-variable growth rate dependence is directly linked to gene expression through a complex regulatory network involving signal cascades that adapt to environmental change (Klumpp et al., 2009; Neidhardt et al., 1990; Schaechter et al., 1958; Scott and Hwa, 2011). This adaptation to the environment means that proteins should be made only when needed. However some genes are expressed

constitutively to enable minimum metabolic activity for survival and DNA repair in the 'waiting' mode before and after the environment change (Fischer and Sauer, 2005). Expression of genes encoding virulence factor regulatory proteins such as the Mga regulon and catabolite control protein CcpA has been shown to be strictly dependent on the growth phase i.e. their maximum expression is with the exponential and the plateau phase *in vitro* (McIver and Scott, 1997; Unnikrishnan et al., 1999). The multifunctional protease *SpeB* (which has C3 degrading activity) is also highly expressed during the exponential growth phase and is strongly affected by culture pH and nutrient availability (Chaussee et al., 1997; Loughman and Caparon, 2006).

1.4 Aims and Hypothesis

Meningeal pathogens cause severe diseases in fish and mammals. Intriguingly, *S. iniae* can cause meningeal symptoms in both humans and fish, yet fish pathogenic Streptococci, even of the same species, have greatly reduced genomes compared with their human pathogenic counterparts (Rosinski-Chupin et al., 2013), narrowing down the potential repertoire of genes that may be critical in causing meningeal infections. There is a trade-off between innate immune resistance that is required for rapid proliferation in the blood and the cellular adhesion that is required to cross the BBB, in that the Capsular polysaccharide (CPS) which appears to be critical for survival in the blood plays no role or impedes cell adhesion. Thus I hypothesized that *(i) S. iniae* possessed alternative systems to CPS that protect it from innate immunity, namely a complement manipulation system which employs C3- and C5a-proteases to corrupt the complement pathway of the host innate immune system, *(ii)* these genes were coordinately regulated with CPS expression to protect against complement and phagocytosis whilst permitting cellular adhesion to allow the development of meningitis.

I employed the gene encoding the priming phosphoglycosyl transferase *cpsE* in the cps operon as a proxy for CPS expression and investigated how this was regulated through the growth phase in comparison to two complement proteases, *cppA* and *scpI*. I investigated coordinate regulation of these genes with the capsular polysaccharide using real time PCR to explore relative expression levels of *scpI* and *cppA* in *S. iniae* along with the initiator critical for CPS expression, *cpsE* through the growth phases in both iron-normal and -limited conditions. I also tested the role of these proteins in cell adhesion by

investigating ability of *S. iniae* to adhere to plastic surface at different points of the growth phase. Finally, I investigated the roles of these proteases in complement resistance by making isogenic knockout mutants in *S. iniae* and testing complement resistance in in vitro assays of whole blood and serum killing.

2. Chapter II. Coordinate inverse regulation of putative complement-degrading proteases scpi and cppa with capsule in fish pathogenic *streptococcus iniae*

2.1 Introduction

Streptococcus species, including *Streptococcus iniae*, are emerging as significant pathogens causing meningitis and septicaemia in diverse hosts ranging from fish to mammals, including man (Agnew and Barnes, 2007). The disease symptoms presented in streptococcus-infected mammals and aquatic species are very similar in spite of reductive evolution of marine strains of Streptococci leading to extensive gene loss (Rosinski-Chupin et al., 2013). This provides an opportunity to explore conserved virulence factors amongst marine and terrestrial strains that may help to elucidate critical pathways in the development of disease symptoms

During infection, bacteria that cause septicemia are exposed to the blood-borne innate immune defences of the host including the complement system and phagocytic cells. During this phase, in Streptococcus species, the polysaccharide capsule that surrounds the bacterial cell is employed to protect them (Doran et al., 2003; Kim et al., 1992; Nizet et al., 1997; St Geme and Falkow, 1991; Unkmeir et al., 2002). Indeed, this capsule is found in almost all virulent strains of *Streptococcus*, and in all rapidly virulent septicaemic isolates (Locke et al., 2007; Millard et al., 2012). The capsule appears to protect against complement either by directly preventing deposition of complement protein C3b on the surface, or simply by being physically too thick to allow penetration of the membrane attack complex (Barnes and Ellis, 2004), which is the end product of the cascade of complement proteins in order to lyse the cell (Ha□nsch et al., 1998; Liszewski et al., 1996). However, capsule deficient isolates of Group B Streptococcus can still enter the central nervous system (CNS) (Doran et al., 2005; Martins et al., 2010; Morona et al., 2006). Moreover, capsule defective variants of *S. iniae* preferentially invade the spinal column of barramundi, but do not cause septicaemia (Millard et al., 2012). This capsule-off status places the bacteria at high risk of lethal attack by complement in the blood stream.

How Streptococcus species translocate into CNS is unknown, but attachment to and translocation across the blood brain barrier must be required, with potential disruption to

the blood brain barrier (BBB). Intriguingly, capsule has been shown to impede cell attachment by other species of streptococcus to cell lines (St Geme and Falkow, 1991). Therefore the bacteria may depress capsule expression in order to attach to the microvascular endothelium of the BBB (Nobbs et al., 2009; Zinkernagel et al., 2008). However, this is also likely to expose the bacteria to lethal risk from the blood-borne innate immune defences. Therefore alternate strategies must be employed to resist these defences in a capsular deficient situation.

The innate immune system inside the central nervous system differs from that of the circulatory system. In contrast to the blood, the unique environment of the CNS is protected by the BBB, composed of microvascular endothelial cells connecting together via very tight junctions and adherent junctions (Schulze and Firth, 1993; Wolburg and Lippoldt, 2002). The cells are surrounded by pericytes, astrocytes, and the endings of neuron cells (Johanson, 1980). This multilayer barrier prevents the access of pathogens and large molecules including immune cells from the circulatory system (Ransohoff et al., 2003). BBB itself can produce complement components for the brain; hence the complement system is still active within the CNS and potentially constitutes an important immune defence in this protected environment (Tauber and Moser, 1999; van Furth et al., 1996). Therefore invasive streptococcus species must retain defence against complement whilst adhering to and translocating across the BBB.

C3 and C5a are two pivotal anaphylatoxins of the complement pathway activating a pro-inflammatory response in both humans and marine fish. Recent studies have indicated a pivotal role for these anaphylotoxins in the onset and development of meningitis (Gasque et al., 1998; Van Beek et al., 2000). The two anaphylatoxins C3a and C5a are the most potent pro-inflammatory mediators and are integral to all three of the complement pathways thus constituting efficient evolutionary targets for interference by proteases of pathogenic bacteria. Interestingly, the corruption of the complement system by the interference from a C5a peptidase and a C3 protease has been demonstrated in *Streptococci* of group A, B, G in their pathogenesis in mammals (Cleary et al., 2004; Hostetter et al., 2004; Ji et al., 1996). Genes encoding these C5a peptidase and C3 protease are retained in fish pathogenic *S. iniae* named *scpI* and *cppA*, respectively (Baiano and Barnes, 2009; Locke et al., 2008; Zhang et al., 2014). C5a peptidase Scp (Streptococcal C5a peptidase) is a branch of the subtilisin family of serine proteases

(Wexler et al., 1985; Wexler and Cleary, 1985), and C3-degrading proteinase belongs to cppA family protein (Okumura et al., 2012; Zhang et al., 2014). The disruption of C5a and C3 results in the slowdown of influx of polymorphonuclear neutrophil leukocytes through failure of chemotactic signalling and neutrophil attraction to the infection site (Beckmann et al., 2002; Hostetter et al., 2004; Locke et al., 2008).

I aim to understand how aquatic streptococcal pathogens manipulate the complement system and capsular expression to determine whether there is a role for complement manipulation in the development of meningitis during fish infection. This will help to identify key pathways in pathogenesis and identify putative surface and secreted targets for future fish vaccines for aquaculture.

2.2 Materials & methods

2.2.1 Bacterial strains and culture conditions

Streptococcus iniae strains QMA0248 and QMA0076 (Aviles et al., 2013) were stored in Todd-Hewitt broth (THB) containing 20% glycerol at -80^{0}C. Isolates were cultured routinely on sheep blood agar plate (Oxoid, Australia) at 28^{0}C for 12-24h. For broth starter cultures, a single colony was used to inoculate Todd-Hewitt broth and incubated at 28^{0}C with gentle agitation for 12-18 h. For growth curves, 30 µl aliquots taken from overnight broth cultures with optical density (OD) at 600 nm adjusted to 1.0, were used to inoculate 1470 µl fresh THB in 24 well tissue culture plates. Growth was recorded by measuring optical density 600 nm over time using a Fluostar Optima Microplate Reader (BMG Labtech, Melbourne). Mid-exponential, late exponential, and stationary phases were determined from at least three replicate experiments with each plate incorporating un-inoculated negative control wells.

2.2.2 Target gene identification

In order to evaluate relative regulation of capsular expression and complement protease expression, genes for C3 protease, C5a peptidase and cpsE (a putative phosphoglycosyl transferase in the capsular operon (Lowe et al., 2007) were chosen. The C5a peptidase coding genes *scpI* of *S. iniae* was retrieved from the sequence of strain SF1 (GenBank CP005941.1). The C3-degrading protease coding gene of *S. iniae* was retrieved by BLAST

analysis using *cppA* from *S. dysgalactiae subsp. equisimilis* against the whole genome sequence of SF1 due to the close phylogenetic relation of these species (Tapp et al., 2003). The resulting amino acid sequences were then analysed for conserved domains by BLAST against the conserved domain database at NCBI (Marchler-Bauer et al., 2015). Putative signal peptides and cleavage sites were predicted using Signal P4.1 (Nielsen et al., 1999a) and molecular weight and pI estimated using ProtParam (web.expasy.org/cgi-bin/protparam/). Next, proteins were modelled using Phyre2 and putative proteolytic active site and metal binding domains identified (Kelley and Sternberg, 2009). Phylogenetic relationships between the proteases and their closest matches by BLAST were inferred by maximum likelihood with Mega 5.2 for Macintosh and supported by bootstrap of 1000 replicates. The putative phosphoglycosyl transferase *cpsE* was as previously published (Lowe et al., 2007).

For normalization of gene expression, three house-keeping genes including DNA gyrase A *gyrA* (Aviles et al., 2013), glutamine synthetase *glnA*, and serine dehydratase *sdhA* were also retrieved from whole genome sequences by BLAST. Primers for qRT-PCR specific for the three target genes and three reference genes were designed with Primer Express 3.0.1 (Applied Biosystems).

2.2.3 Determination of gene expression with respect to growth

Each strain was cultured in 24-well plates with three biological replicates in THB as described above. The sample points were mid-exponential phase, late exponential phase, and early stationary phase. At each sample point, 500 µl of sample was taken from each of 3 replicate wells per strain per treatment for optical density measurement at 600nm, and the cells from the remaining 1ml were immediately pelleted at 10,000 x g for 2 min at room temperature and re-suspended in 1ml of TRIsure ^{TM} (Bioline US Inc) and transferred to a 2 ml screw-capped tube containing MagNA Lyser Green Beads (Roche Diagnostics, Germany) then homogenized at 7,000 rpm for 40s in a Roche MagnaLyser and immediately chilled on ice. The lysate was centrifuged at 6000 x g for 3 min to pellet the beads and cell debris then the supernatant was transferred into the new tube. After adding 200µl chloroform, the lysate was shaken vigorously for 15s, incubated at room temperature for 2 min then centrifuged at 12,000 x g for 15 min at 4^0C. The aqueous phase was transferred carefully to a new tube, and the RNA was precipitated by adding

500 µl of isopropyl alcohol followed by centrifugation at 10,000 x g for 10 min at 4^0C. The RNA pellet was washed with 1ml of ethanol 75% and air-dried for 3h. 30 µl of DNase- and RNase-free water was added to re-suspend the pellet followed by storing at -80^0C for further study.

Genomic DNA was digested using the RNAse-free DNAse set (Qiagen), RNA was quantified fluorimetrically using a Qubit fluorimeter (Life Technologies, Melbourne) and 20ng per sample was used for cDNA synthesis with the QuantiTect® Reverse Transcription Kit (Qiagen®) in accordance with the manufacturer's instructions.

All real-time PCR experiments were performed in 384 well plates with reagents dispensed using an epMotion 5075 Robot (Eppendorf, Hamburg) and thermal cycling was performed with the ABI ViiA7 using SYBRGreen Master Mix (Applied Biosystems). The primers and input cDNA concentrations were optimized at 200nM and 1ng respectively per reaction to yield ~100% efficiency. In each reaction there were 0.2 µl of Forward primer, 0.2 µl of Reverse primer (both from 10 µM stock solutions), 5 µl of SYBRGreen Master Mix, 2 µl of cDNA 0.5ng/µl, and 2.6 µl of water. Cycling parameters were: 95^0C for 10 min followed by 40 cycles of 95^0C for 15s and 62^0C for 1min, then a final melt curve at 95^0C for 15s, 60^0C for 1min and 95^0C for 15s. All temperature cycling was performed with acceleration at 1.6^0C/s.

2.2.4 Determination of plastic adherence with respect to growth phase

Streptococcus iniae QMA0076 and QMA0248 were grown in THB at 28^0C over night to reach the stationary phase, then adjusted to OD_{600} ~ 1 and proceeded to the plastic adherence assay. Three cultures per strain were inoculated with a 2% v/v overnight culture in THB and incubated at 28^0C. These cultures were stopped at the desired time corresponding to mid-exponential, late-exponential, and stationary phases. The optical densities of these cultures were measured at 600nm and the OD of all cultures adjusted by dilution to be equivalent to the mid-exponential value. An aliquot (100 µl) of each sample transferred to the wells of a U-bottom 96-well plate (10 wells per treatment as technical replicates) and the cells brought into contact with the plastic by centrifugation at 500 x g for 10 min. Plates were incubated at 28^0C for 0, 15 and 30 min then the supernatant was removed and plates were washed twice with water followed by addition of 100 µl 0.05%

Coomassie blue in 40% methanol, 10 % acetic acid at room temperature (RT) for 15min. Subsequently, the stain was removed and plates were washed with water three times before adding 200 µl of 40 % methanol, 10% acetic acid and incubating at RT for 15 min to resolubilise the bound dye prior to reading optical density at 620nm.

2.2.5 Whole blood and serum bactericidal assays

Starter cultures of *S. iniae* QMA0076 and QMA0248 were grown in THB overnight at 28^0C with shaking. *Escherichia coli* DH5α was also cultured as a susceptible control. Cell densities were adjusted to OD_{600} = 1, inoculated at 2% v/v into THB and incubated at 28^0C with gentle agitation. These cultures were then stopped at the time corresponding to the mid-exponential, late-exponential, and stationary phases. OD_{600} was then adjusted for each culture to the same optical density as the mid-exponential culture by dilution. Each sample was then diluted a further $2x10^4$ times for the assay. Blood from juvenile barramundi was collected fresh as required aseptically by caudal venepuncture in heparinised syringes and diluted 1:1 in RPMI medium. Aliquots (50 µl) were distributed into the wells of a sterile 96-well U-bottom culture plate and 50 µL of each standardised cell suspension was added into 5 replicate wells per treatment (bacterial isolate and time point in the growth curve). The mixture was incubated at 28^0C for 30 min. Uninoculated blood was also incubated to ascertain sterility. Bacteria harvested at each of the three growth phases were incubated in THB (ie. No blood or serum) as normal growth controls. Number of surviving cells was determined by viable counting on THA.

For serum bactericidal assays, the same protocols were followed with the following adjustments. In place of blood, heat-inactivated normal serum, heat-inactivated *S. iniae* QMA0248-anti serum prepared previously (Aviles et al., 2013), heat-inactivated normal serum supplemented fresh normal serum, and heat-inactivated *S. iniae* QMA0248-anti serum supplemented with fresh normal serum. Heat inactivation was by incubation at 56^0C for 30 min to inactivate all the complement proteins. The fresh normal serum was used as a homogeneous source of complement proteins between the assays. The four sera were diluted 1:1 with RPMI media and 100 µl distributed into the wells of a 96-well U-bottom plate with 15 µl of bacterial suspension as above and incubated at 28^0C for 30 min prior to enumeration by viable counting on THA. All assays were repeated three times and representative data are presented.

2.2.6 Data analysis

PCR efficiency for each primer set was determined with serial dilutions of cDNA samples from the four strains. The PCR exponential amplifications of these genes were calculated based on the slope estimated by Ct value of a serial dilution in the validation experiment against Log_{10} of the cDNA amount per reaction, described by $E = 10^{-1/slope}$ (Hellemans et al., 2007). Any variation in amplification efficiency was then accounted for during normalization of relative gene expression differences between the target group and the control group. The normalization factor was calculated based on the geometric mean of the relative quantities valued for three reference genes using REST (Pfaffl et al., 2002; Vandesompele et al., 2002). Ct data were analysed with REST based on the Pair Wise Fixed Reallocation Randomisation Test (Pfaffl et al., 2002), in which the normalized relative quantities were calculated based on the ratio of the group means for target gene against the normalized reference genes. The result represented the up- or down-regulation of the target group (i.e. late-exponential, or early stationary phase) compared to the control group (i.e. mid-exponential phase).

Bacterial growth curves were analysed by non-linear regression based on the Gompertz equation (Mytilinaios et al., 2012). Differences in susceptibility of bacteria in whole blood and sera was analysed by t-test using Prism 6 for Macintosh (GraphPad Inc, California).

2.3 Results

2.3.1 *ScpI* and *cppA* are highly a conserved peptidase and metalloprotease respectively

ScpI is a subtilisin-like serine protease comprising a pre-protein of 1122 aa with a predicted double arginine bacterial signal peptide cleavage site at residues 32 and 33. The mature peptide has a predicted molecular weight of 120 KDa and a pI of 4.86. There is a highly conserved c5 S8 peptidase domain between residue 65 and 539 of the mature monomer. A proline, leucine leucine polypeptide binding site was conserved with *S. pyogenes* C5a peptidase along with an active site triad at residues Asp114, His181 and Ser501 (82, 149 and 469 in the mature monomer)(Figure 8a). Two putative transmembrane helices were identified between residues 468-482 and 1070-1083, close

to the C terminus by Phyre2, but only the C terminus transmembrane helix was supported by the other two prediction models (TMHMM and psipred)(Figure 8a).

CppA is small protein estimated at 28.1 KDa with two Glo-EDI-BRP-like metalloprotease active domains (Figure 8b). BLAST search revealed closest relationship with peptidase from *S. parauberis*, the C3-degrading proteinase from *S. dysgalactiae*, and the CppA N-terminal domain from *S. pyogenes* (Figure 8b). No signal peptide was detected, nor were any potential transmembrane helices, beta barrel or Gram positive membrane anchor predicted (Figure 8b).

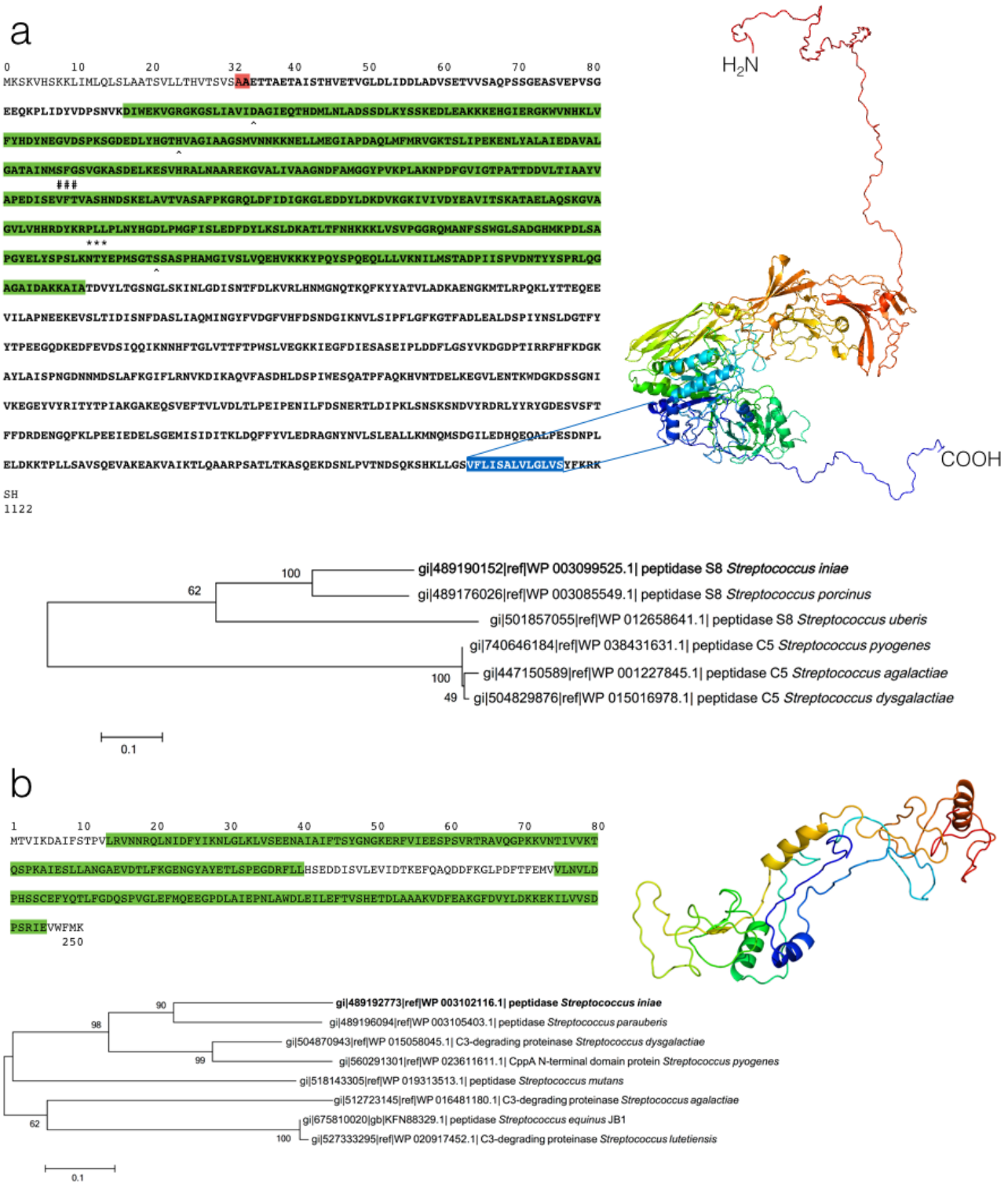

*Figure 8. Amino acid sequences showing key features, predicted structure and phylogenetic position for scpI (a) and cppA (b). Conserved functional domains are highlighted in green; a) Peptidase S8 family domain in Streptococcal C5a peptidases and b) Glo_EDI_BRP_like metalloprotease domains. The transmembrane helix in scpI is highlighted in dark blue. ^Catalytic triad residues; #putative polypeptide binding site; *Putative active site. Phylogenetic relationships were inferred by maximum likelihood with the closes matches by BLAST. Bootstrap support values are indicated.*

2.3.2 *ScpI* and *cppA* are coordinately but inversely regulated with *cspE* through the growth phases

In order to investigate whether cps and the putative complement proteases are coordinately regulated, the effect of growth phase on expression was considered. First, growth curves were constructed for each of the bacterial isolates under the various culture conditions. From the curves potential transition points were identified for comparison of gene expression. Transition from log to stationary phase was identified in each curve and samples taken during mid-log as a reference, and then either side of point of inflexion in the growth curves (Appendix A).

Expression of target genes was normalised against 3 reference genes and levels of gene expression were determined relative to expression during mid-log growth. Changes in expression are presented as Log_2-fold changes (Figure 9). *Streptococcus iniae* isolates showed switching of gene expression between mid-log and late log, with *scpI* and *cppA* up-regulated approximately 2 fold whilst *cpsE* expression was downregulated 2 fold (Figure 9A). Once into stationary phase there was a second switching of expression, this time with *cpsE* up-regulated almost 8-fold in strain *S. iniae* QMA0248 and 2 fold in strain *S. iniae* QMA0076 (Figure 9). In contrast the expression of *scpI* was strongly down regulated with 2 fold reduced expression in strain *S. iniae* QMA0248 whist expression was down regulated more than 4 fold in strain QMA0076. There was no significant change in expression of *cppA* at this time (Figure 9).

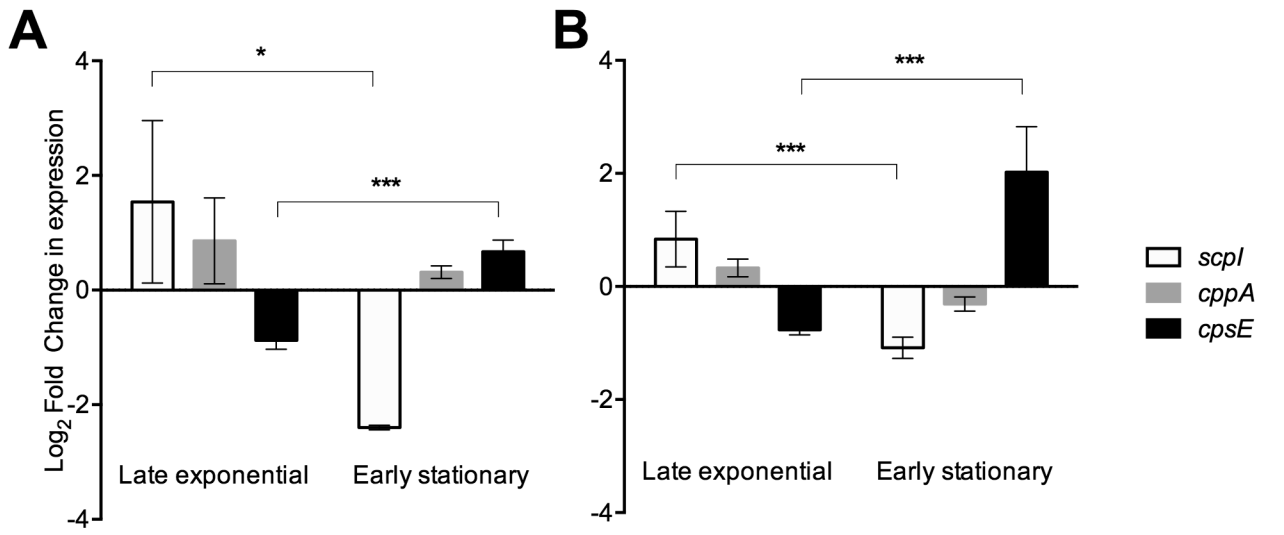

*Figure 9. Expression (normalized against three reference genes) of scpI, cppA, and cpsE at late exponential and stationary phase relative to mid-exponential phase in Todd-Hewitt Broth. (A)QMA0248, (B) QMA0076 (Mean ± SD, n = 9; *p<0.05; ***p<0.001)*

2.3.3 Cell adherence is affected by growth phase

The adherence of *S. iniae* to plastic following 15 min incubation (< 1 generation time) was determined in 96 well plates and was consistent between isolates (Figure 10). The number of adherent bacteria was plotted relative to the mid-exponential value to determine how adherence changed with the growth phase of the inoculum. There was significant increase in adherence between mid and late log phase, whilst adherence decreased significantly in stationary phase (Figure 10).

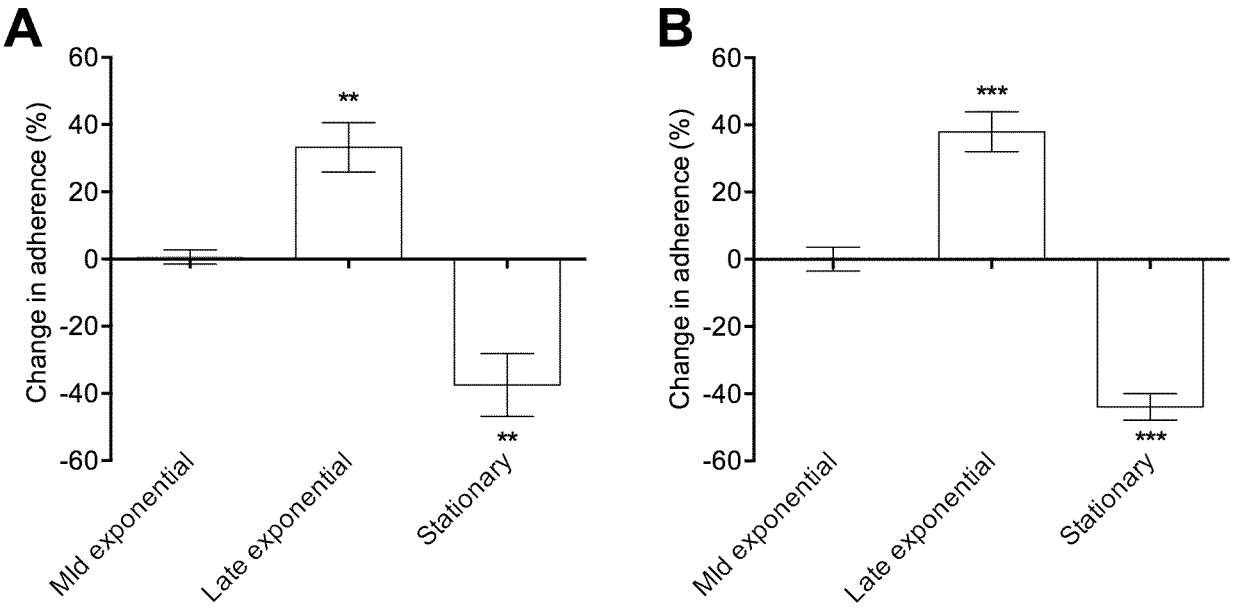

*Figure 10. Surface adherence of S. iniae to plastic at different phases of growth. (A) S. iniae QMA0248, (B) S. iniae QMA0076. (Mean ± SD, n = 3; **p<0.01; ***p<0.001)*

2.3.4 *S. iniae* growth and survival in whole blood and serum is affected by growth phase of the inoculum

The susceptibility of bacteria from each sample point in the growth phase to whole blood killing in terms of percentage survival after 30 min incubation was determined (Figure 11). The susceptibility pattern of *S. iniae* was consistent between both strains. The mid-exponential sample went through one doubling in population during the 30 min incubation in whole blood whilst the late exponential sample was significantly killed (Figure 11). Cells from stationary phase did not grow in the blood within the 30 min incubation employed during the assay (Figure 11). The control *E. coli* DH5a was killed by 30 min incubation in whole barramundi blood (not shown).

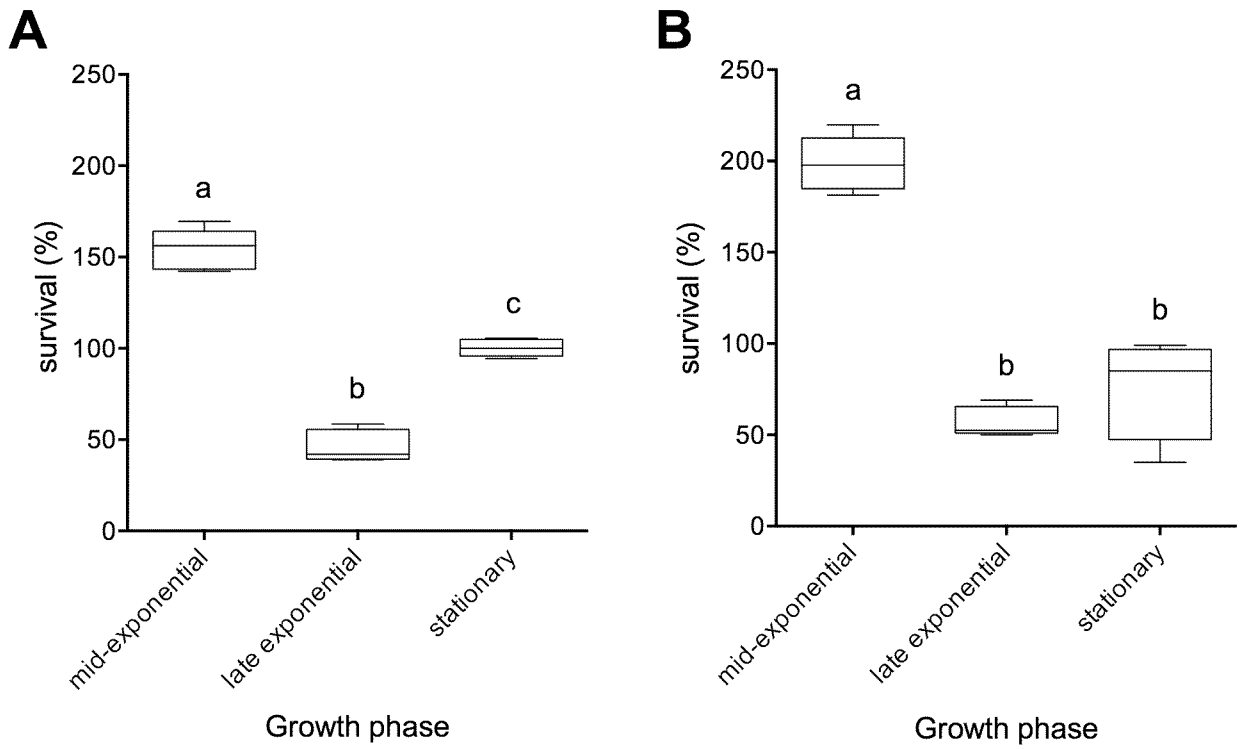

Figure 11. Survival of S. iniae QMA0248 (A) and QMA0076 (B) over 60 min in whole barramundi blood when a standardised number of cells from different growth phases are used as the starting inoculum. The survival percentage of each growth phase was estimated based on the number of colony forming units before and after incubation for 60 min at 28°C (Mean ± SD, n = 3; significant difference indicated by different letters, p<0.05).

The ability of *S. iniae* strain QMA0248 to grow in serum and antiserum was analyzed with and without complement proteins by supplementing heat inactivated sera with fresh normal serum as a source of heat labile proteins (Figure 12). In heat inactivated normal serum, cells harvested at mid-exponential phase grew, approximately doubling in 30 min incubation (Figure 12). Cells harvested later in the growth phase did not divide in serum over the 30 min period. Presence of specific antibody appeared to inhibit growth in serum using mid-exponential inoculum, and there was significant killing of bacteria by antiserum when the cells used in the inoculum were harvested at mid or late exponential phase (Figure 12), regardless of the phase of growth or the (Figure 12). Surprisingly, addition of fresh serum increased growth in both normal serum and antiserum, with only cells harvested in stationary phase either inhibited or killed (Figure 12).

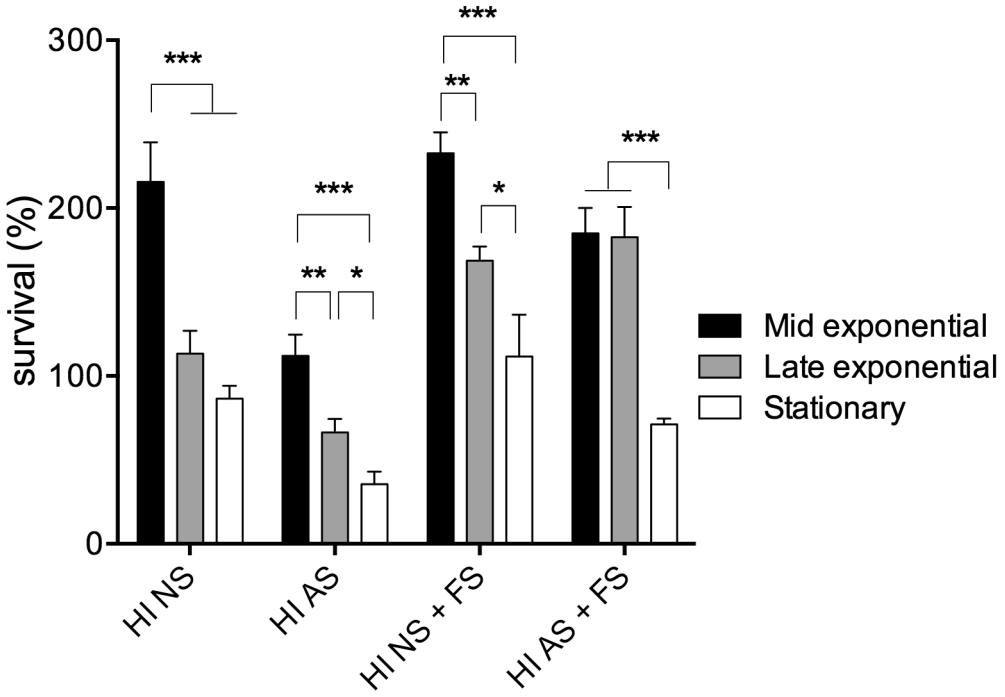

*Figure 12. Survival of S. iniae QMA0248 in barramundi serum using inocula from different growth phases. HINS: heat-inactivated normal serum, HIAS: heat-inactivated anti-QMA0248 antiserum, HINS+FS and HIAS+FS: heat-inactivated normal serum or antiserum with fresh serum added as a uniform source of complement and other heat-labile plasma proteins (Mean ± SD, n = 3; *p<0.05; **p<0.01; ***p<0.001)*

2.4 Discussion

Bacterial pathogens that cause septicaemia, such as the fish pathogen *S. iniae* must survive and replicate in the blood in order to disseminate through the host and therefore must be resistant to a range of host innate defences. Many bacteria that cause septicaemia express polysaccharide capsules as key virulence factors (Cunningham, 2000; Glauser et al., 1991), including *S. iniae* (Barnes et al., 2003). However, resistance mechanisms such as extracellular polysaccharide capsule may lead to a trade-off in ability to attach and invade into other organs and tissues (Hammerschmidt et al., 1996; St Geme and Falkow, 1991; Vogel et al., 1996). This is counter to the pathology of *S. iniae* which invades the central nervous system and therefore must attach and traverse the blood brain barrier, implicating down-regulation of CPS during invasion, which may leave the pathogen susceptible to host defences. Here I investigated whether CPS expression in *S. iniae* is coordinately regulated with alternate factors that may protect from innate immune defence in the absence CPS expression. I employed the gene *cpsE,* a putative transferase in the

cps operon, as a proxy for CPS expression and investigated how this was regulated through the growth phase in comparison to two putative complement proteases, *cppA* and *scpI*. In *S. iniae cps* expression is regulated through the growth phase with down regulation towards the end of exponential growth and up-regulation once in stationary phase where rates of cell death and cell division are equal. Interestingly, the C5a protease gene *scpI* was inversely regulated suggesting potential coordinate regulation to provide protection against complement activity when CPS expression is low, perhaps permitting improved attachment at appropriate triggers without sacrificing complement resistance. It is highly likely that multiple interacting factors effect regulation of these systems during growth phase; for example elevated CO_2, superoxide level, oxygen level, and other metal ions such as zinc or manganese (Kreikemeyer et al., 2003). Further work is required to determine what factors in the host may regulate this switch.

The susceptibility of *S. iniae* to host humoral innate immunity over the growth phases was investigated in vitro by whole blood and serum killing assay. Although the susceptibility of *S. iniae* to whole blood killing over the growth phases was varied, the general pattern was consistent between strains (Figure 11). Generally, mid exponential phase inoculation resulted in growth in blood over the incubation period. This is largely in agreement with previous research in which virulent *S. iniae* recovered from mid-exponential culture grew rapidly in whole blood, increasing approximately 5-fold after 1 h incubation (Buchanan et al., 2008). However, inhibition of growth of *S. iniae* by whole blood has also been reported in which the survival of mid exponential *S.iniae* strain 9033 was only 77% after 1.5 h of incubation (Fuller et al., 2001), these apparently contrasting observations may result from the difference in the incubation time that provided the bacteria enough time to transform to the next growth phase. This hypothesis is supported by our results, in that the survival of the late log sample dramatically declined from high resistance detected in the mid log sample. The reduction of the ability to avoid phagocytosis when the capsule is down-regulated is also consistent with previous studies that a capsule deficient mutant was susceptible to the blood (Locke et al., 2007). In the serum bactericidal assay resistance to serum killing generally declined over the growth phases. In heat inactivated antiserum, growth was restricted regardless of the growth phase of the inoculum, compared to heat-inactivated normal serum. Surprisingly, when specific antiserum was supplemented with fresh serum as a source of complement and other heat-labile plasma proteins, growth/survival increased compared to heat inactivated antiserum alone, regardless of

growth phase of the inoculum. Streptococcal complement proteases can have multiple functions. The C3b-degrading proteinase SpeB in GAS can interact with a broad range of molecules including cleavage of antibodies at the hinge region once they bind to antigens on the surface of *S. pyogenes* (Söderberg et al., 2008). Furthermore, this protease can also hydrolyse and destroy chemokines, which are secreted by inflamed epithelial cells, and are responsible for signalling and antibacterial activity (Egesten et al., 2009). It can also induce inflammation by cleaving pro-interleukin-1β into active form interleukin-1β (Kapur et al., 1993a), hydrolysing H-kininogen into bradykinin (Herwald et al., 1996), inducing histamine release and mast cells degranulation (Watanabe et al., 2002), which enhances *S. pyogenes* infection. It is likely SpeB is an important part of the multi-target invasion strategy of *GAS a*s it effectively degrades innate immune barriers including fibrin/fibrinogen in blood coagulation (Elliott, 1945; Matsuka et al., 1999), antigen-bound plasmin (Svensson et al., 2002), extracellular matrix proteins vitronectin and fibronectin (Kapur et al., 1993b); and indirectly participates in tissue degradation and apoptosis (Burns et al., 1996; Tamura et al., 2004). In terms of complement pathway corruption, mature SpeB can cleave the C3 component and prevent the formation of the C5 convertase complex (Kuo et al., 2008; Terao et al., 2008). The cleavage of C3 leads to failure of opsonizing bacteria by C3b, which prevents the formation of extreme pro-inflammatory mediator C5a, the assembly of MAC and ultimately phagocytosis by neutrophils. In addition, mature SpeB also can lyse Properdin, a protein that enhances the stabilization of the C5 convertase complex in the alternative pathway (Tsao et al., 2006) leading to a labile complex, which is subsequently inactivated. Therefore, these complement proteases in Streptococcal species do not only possess C5a and C3 cleavage ability, but also interact with other molecules to escape phagocytic clearance. Alternate functions of *scpI* and *cppA* require further investigation in fish models

In the blood, virulent Streptococcus cause septicaemia, but capsule-deficient mutants preferably enter CNS instead of remaining in the blood stream (Doran et al., 2005; Martins et al., 2010; Millard et al., 2012; Morona et al., 2006). The successful adherence and invasion of meningeal pathogens with ineffective capsules (Doran et al., 2005; Hook et al., 1975; Monsinjon et al., 2001; Nizet et al., 1997; Nonaka et al., 1985) shows that pathogens have an alternative strategy, including potentially complement proteases, which protects them from host phagocytosis and other immune defences, C3-degrading protease *cppA* has been reported in *S. iniae* (Zhang et al., 2014), *S. pneumoniae* (Angel et al.,

1994), *S. dysgalactiae* subsp. *dysgalactiae* (Rato et al., 2011) and it can degrade and inactivate soluble C3. C5a peptidase Scp can cleave C5a at the binding site for polymorphonuclear leukocytes, resulting in the slowdown of influx of polymorphonuclear neutrophil leukocytes through failure of chemotactic signalling and neutrophil attraction to the infection site (Angel et al., 1994; Cleary et al., 2004). However, the up regulation of *scpI* during late exponential phase in this study did not fully protect *S. iniae* in whole blood, where neutrophils are likely to be the major means of killing activity. This role for neutrophils is supported here as *S. iniae* from late exponential phase were able to grow in cell free HI-NS and HI-NS + fresh serum suggesting high resistance to complement and other humoral factors. Both Group A and B Streptococci possess the mechanism to escape from phagocytosis, and clearance of GAS at the infection site is delayed when Scp is expressed on its surface (Hung et al., 2012). Additionally, Scp acts as an adhesin to Fibronectin type III and enhances invasion to epithelial cells (Beckmann et al., 2002). Interestingly, anti-ScpB antibody opsonizes GBS across serotypes, resulting in increased killing rate and macrophage oxidative burst (Cheng et al., 2001).

In order to tease out the roles of *scpI* and *cppA* in *S. iniae* virulence, knockout mutants should be prepared. Indeed an *scpI* knockout has been investigated previously and did not reduce virulence in hybrid striped bass challenge model (Locke et al., 2008). It is intriguing to speculate why this gene should be retained, and functionally regulated if it plays no role in survival in the host. As the challenge models in the previous study were by intraperitoneal (IP) and intramuscular (IM) injection, it may be that *scpI* is required early as it translocates from the environment into the host. Moreover, the inoculum for the phenotypic assays in the Locke's study was at the mid-log phase where *scpI* is down regulated. In addition, *cppA* may inactivate the complement system upstream of the C5a peptidase (Locke et al., 2008), and I have shown here that these genes are regulated together over the growth phases. However, I remain to be convinced that *cppA* is a complement degrading protease due to its predicted cytoplasmic location, although *in silico* predictions have limitations. Therefore, dual and single knock out isogenic mutants should be investigated in virulence models to determine their role in pathogenesis.

In conclusion, coordinate inverse regulation of cps and complement proteases appears to occur in marine pathogenic *S. iniae* with regulation occurring by growth phase. However

phase regulation is multifactorial and complex. Further investigation is required to determine specific triggers of this regulation that may apply in the host during infection.

3. Chapter III. Role of complement proteases in fish pathogenic *Streptococcus iniae*

3.1 Introduction

In the previous chapter, the susceptibility of wild type *S. iniae* to host humoral innate immunity over the growth phases was investigated in vitro by whole blood and serum killing assay. Generally, mid exponential phase inoculation resulted in growth in fresh barramundi blood over the incubation period, while an inoculum derived from late-exponential phase was susceptible to killing (Chan N. Dieu, 2015). To determine whether neutrophils or plasma factors were involved I also investigated growth and survival in cell free serum in the presence and absence of specific antibody and heat labile plasma factors. The results were complex showing an effect of antibody in heat inactivated serum but, surprisingly, increased growth in serum when fresh serum was added as a source of complement (Chan N. Dieu, 2015). To further elucidate the trade off between complement proteases and capsule, particularly during late-exponential phase of growth, I prepared isogenic knock out mutants of both proteases in our virulent model strain *S. iniae* QMA0248. An *scpI* knockout has been investigated previously and did not reduce virulence in hybrid striped bass challenge model (Locke et al., 2008): Indeed, Locke's Δ*scpI* mutant of strain K288 conserved the phenotypic properties of the wild type *S.iniae* including the coccoid morphology, cell buoyancy and cell surface charge, and haemolytic activity against fish red blood cells. However, K288ΔscpI produced a higher proportion of multimeric cocci chains than the wild type (Locke et al., 2008). The precise in-frame allelic replacement of *scpI* in K288 did not reduce virulence in a hybrid striped bass IP challenge model ($p=0.31$), but was possibly involved in the change in killing rate in the intramuscular injection (IM) challenge model ($p<0.01$). In the zebra fish IM challenge model, although the mortality following challenge with K288ΔscpI occurred later compared to fish challenged with the wild type, there was no significant difference in final mortality ($p=0.985$). Therefore, these authors concluded *scpI* was not required for virulence in fish when delivered by injection (Locke et al., 2008). This lack of conclusive phenotype in the in vitro phenotypic assays may result from the inoculum in Locke et al 's study being mid-log phase where *scpI* is not highly expressed relative to either capsule or reference genes (Chan N. Dieu, 2015). Moreover, IP- and IM-injection routes in Locke's challenge model

might ignore the role of ScpI during the early stages of translocation from the environment. The role of Scp in the inhibition of leukocyte chemotaxis has been reported in other Streptococci (Cleary et al., 1992; Hill et al., 1988). Additionally, the effect of *scpI* deletion may be masked by the activity of C3-degrading protease CppA which inhibit complement deposition and signalling further upstream from C5a (Locke et al., 2008). Indeed, I have shown that the expression of these two proteases is regulated together over the growth phases (Chan N. Dieu, 2015). Therefore, further study of knock out mutants of these genes is warranted to understand the roles of these complement proteases in resistance to the host innate immune system in vitro and how their expression may compensate for down regulation of capsule during the attachment phase of infection.

3.2 Materials and methods

3.2.1 Bacterial strains and culturing

S. iniae strains QMA0248 and QMA0076 (Aviles et al., 2013) were stored in Todd-Hewitt broth (THB) containing 20% glycerol at -80^{0}C. Isolates were cultured routinely on sheep blood agar plates (Oxoid, Australia) at 28^{0}C for 12-24h. For broth starter cultures, a single colony was used to inoculate Todd-Hewitt broth and incubated at 28^{0}C with gentle agitation for 12-18 h.

3.2.2 Isogenic knock-out mutants

Isogenic knock-out mutants Δ*scpI* and Δ*cppA* in QMA0248 were prepared by allelic exchange mutagenesis using the spectinomycin resistance (*aad9*) gene from pBBR1MCS-5 (LeBlanc et al., 1991) (Figure 13). Primers for PCR were designed to amplify ≈1000 bp upstream and ≈1000 bp downstream of *S. iniae* chromosomal DNA flanking regions of the *scpI* and *cppA* genes. The 3'-end of the reverse primer of the upstream flanking region and the 5'-end of forward primer of the downstream flanking region contained a 3' overhang and 5' overhang respectively consisting of 25 bp complementary to the 5' region or 3' region of the *aad9* spectinomycin resistance gene. The primer sequences are listed in the Appendix B.

The upstream and downstream fragments were combined with the 753 bp *aad9* gene by a three-way fusion PCR. The fusion PCR reaction comprised 2 µl of 50mM $MgSO_4$, 10 µl of

5X PCR Buffer (Platinum *Pfx* kit, Invitrogen), 10 µl of 10mM dNTPs, 200 ng of upstream fragment, 200 ng of downstream fragment, 200 ng of *aad9* fragment, brought to a volume of 43.5 µl with sterile ultra-pure water. Fusion of fragments was initiated at 94^0C for 3 min, followed by 10 cycles of 94^0C for 1 min, 45^0C for 2 min, 72^0C for 3 min, and cooling to 10^0C. After these 10 cycles, 1 µl of 10µM 5'- upstream flanking region forward primer, 1 µl of 10µM 3'- downstream flanking region reverse primer, 2 µl of DMSO, and 0.5 µl of Platinum *Pfx* polymerase were added. Then further cycling was performed at 94^0C for 3 min, followed by 35 cycles of 94^0C for 1 min, 52^0C for 30 s, and 68^0C for 5 min. Final extension was carried out at 68^0C for 10 min. The fusion product was gel-purified and cloned into a pCR™II-Blunt-TOPO vector (Invitrogen™) and chemically transformed into TOP10 *E. coli* competent cells (Invitrogen™) following the protocol supplied with the Zero Blunt TOPO PCR Cloning Kit. Positive clones were selected on LB-Kanamycin (50µg/ml) plate and submitted to colony PCR and sequencing to confirm integrity of the fusion product.

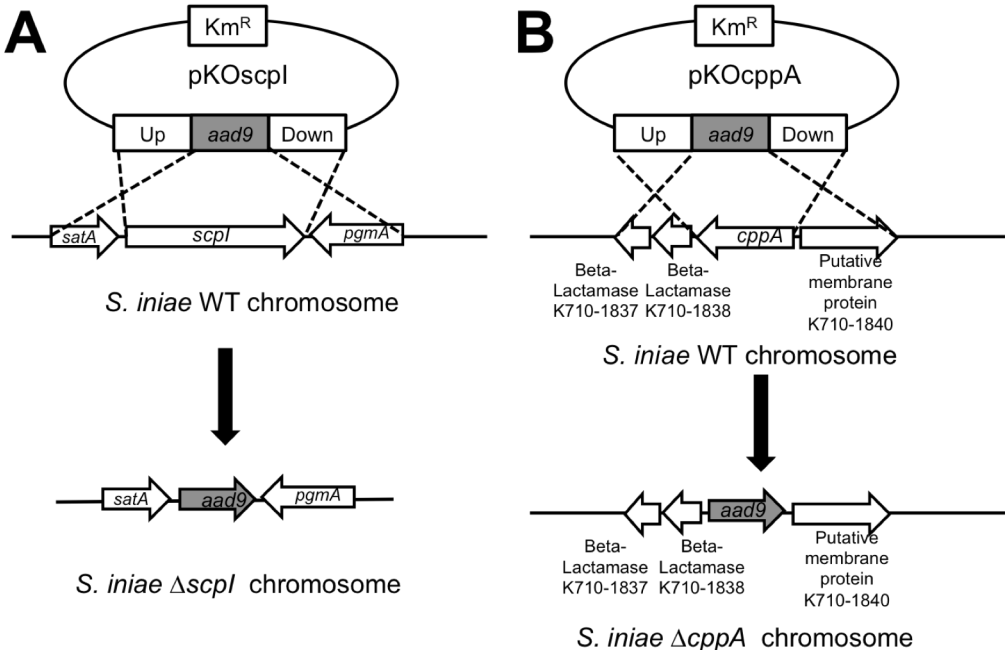

Figure 13. Isogenic mutagenesis knock out mutants diagram of (A) scpI and (B) cppA in S. iniae. Spectinomycin encoding gene aad9 was introduced into the wild type genome of S. iniae by allelic exchange mutagenesis between the homologous up- and down-stream flanking regions. (Locke et al., 2008).

Plasmid DNA from the positive transformants was isolated using a modification of the alkaline lysis method previously described (Birnboim, 1983). Briefly, positive colonies were grown at 37^0C overnight in LB supplemented with Kanamycin 50 µg/ml and cells were harvested and re-suspended in 100 µl of re-suspension buffer comprising 50mM glucose, 10mM EDTA, 25mM Tris at pH 8.0. The suspension was lysed with 200 µl of lysis buffer (0.2N NaOH and 1% SDS), neutralized with 200 µl of 3M Potassium acetate at pH 6.0. The mixture was not vortexed to avoid disrupting the chromosomal DNA. The lysate was precipitated by adding 1ml isopropanol followed by incubating at -80^0C for 30 min to enhance the DNA precipitation. The plasmid was harvested by centrifuging at 13,500 rpm in an Eppendorf minispin at 4^0C for 5 min, subsequently washing with 70% EtOH followed by harvesting at 13,500 rpm at 4^0C for 3min in an Eppendorf minispin centrifuge. The plasmid was air-dried and re-suspended in 50 µl TE. Two hundred micrograms of recombinant plasmid was linearized by digestion with 20 units of restriction enzyme PstI at 37^0C for 12 h then purified by salting out with 50 µl of 3M Sodium acetate. The plasmid was then precipitated with 2ml isopropanol followed by incubating at -80^0C for 30 min and harvesting by centrifuging at 13,500 rpm in an Eppendorf minispin at 4^0C for 5 min, subsequently washing with 70% EtOH followed by harvesting at 13,500 rpm at 4^0C for 3min in an Eppendorf minispin centrifuge.

Ten micrograms of the linearized knockout plasmid was introduced to *S. iniae* QMA0248 competent cells by electroporation (Framson et al., 1997; Locke et al., 2007). The colonies that contain pKO*scpI* (or pKO*cppA*) were identified by spectinomycin selection (100µg/ml) on THA. Successful knockout mutation was confirmed by colony PCR and sequencing of the targeted in-frame replacement of *scpI* (or *cppA*) by *aad9* gene (Figure 13). Knockout mutants were employed in serum and whole blood killing assays as described below.

3.2.3 β-hemolysis sheep red blood cells

The hemolysis assay was performed essentially as previously with minor modification (Locke et al., 2008). The sheep whole blood (Oxoid, Australia) was centrifuged at 500 x g for 10 min at 4^0C. Subsequently, 1ml of pellet was re-suspended with 3ml of HEPES-NaCl wash solution (HEPES 20mM, NaCl 0.9%) and cells collected at 1000 x g for 30 min at 4^0C followed by discarding the supernatant. This washing step was repeated three times or until the supernatant became clear. Red blood cells (RBCs) were then centrifuged at

1000 x g for 5 min at 4^0C to pack the cells then a 2% (v/v) working suspension was prepared by adding 100 µl of packed cells into 4.9 ml phosphate buffered saline (PBS). In a 96-well U-bottom microtitre plate, 150 µl of mid log culture of *S. iniae* QMA0248 wild type, ∆*scpI* and ∆*cppA* was deposited into each well with 10 replicates per isolate, to which 150 µl of the RBC suspension was subsequently added. The positive control of completely lysed cells comprised 148.5 µl of THB, 148.5 µl of RBCs 2%, and 3 µl of Triton X-100, whilst the blank contained 150 µl THB and 150 µl RBC suspension. The plate was incubated at 28^0C for 2 h then at 4^0C for 2 h, followed by centrifugation at 1,500 x g for 5 min. An aliquot (200 µl) from each well was transferred to a new 96-well plate and the optical density was read at 405 nm.

3.2.4 Survival of Δ*scpI* and Δ*cppA* in whole barramundi blood

Starter cultures of *S. iniae* QMA0248 wild type, Δ*scpI* and Δ*cppA* were grown in THB overnight at 28^0C with shaking. *E.coli* DH5α was also cultured as a susceptible control. Cell densities were adjusted to OD_{600} ~ 1, inoculated at 2% v/v into THB and incubated at 28^0C with gentle agitation. These cultures were then stopped at the time corresponding to mid-exponential and late-exponential phases. OD_{600} was then adjusted for each culture to the same optical density as the mid-exponential culture by dilution. Each sample was then diluted a further $2x10^4$ times for the assay. Blood from juvenile barramundi was collected fresh as required aseptically by caudal venepuncture in heparinised syringes and diluted 1:1 in RPMI media. Aliquots (50 µl) were distributed into the wells of a sterile 96-well U-bottom culture plate and 50 µL of each standardized cell suspension was added into 5 replicate wells per treatment (bacterial isolate and time point in the growth curve). The mixture was incubated at 28^0C for 30 min. Uninoculated blood was also incubated to ascertain sterility. Number of surviving cells was determined by viable counting on THA.

3.2.5 Susceptibility of Δ*scpI* and Δ*cppA* to killing by serum and antiserum

For serum bactericidal assays, the same protocols were followed with the following adjustments. In place of blood, heat-inactivated normal serum, heat-inactivated *S. iniae* QMA0248-anti serum prepared previously (Aviles et al., 2013), heat-inactivated normal serum supplemented fresh normal serum, and heat-inactivated *S. iniae* QMA0248-anti serum supplemented with fresh normal serum. Heat inactivation was by incubation at 56^0C

for 30 min to inactivate all the complement proteins. The fresh normal serum was used as a homogeneous source of complement proteins between the assays. The four sera were diluted 1:1 with RPMI media and 100 μl distributed into the wells of a 96-well U-bottom plate with 15 μl of bacterial suspension as above and incubated at 28^0C for 30 min prior to enumeration by viable counting on THA. All assays were repeated three times and representative data are presented.

3.2.6 Statistical analysis

Differences in haemolytic activity were analysed by one-way ANOVA, and differences in susceptibility of bacteria in whole blood and sera were analysed by two-way ANOVA using Prism 6 for Macintosh (GraphPad Inc, California).

3.3 Results

3.3.1 Hemolytic activity of knock-out mutants Δ*scpI* and Δ*cppA* was lower than wild type

Although the haemolytic activity of knock out mutants was lower than wild type, their values ranged broadly and some samples were equal to or higher than the wild type (Figure 14). The haemolytic activities of two knock out mutants were similar and their differences compared with wild type were marginally non-significant (p=0.0575 and p=0.0594, respectively).

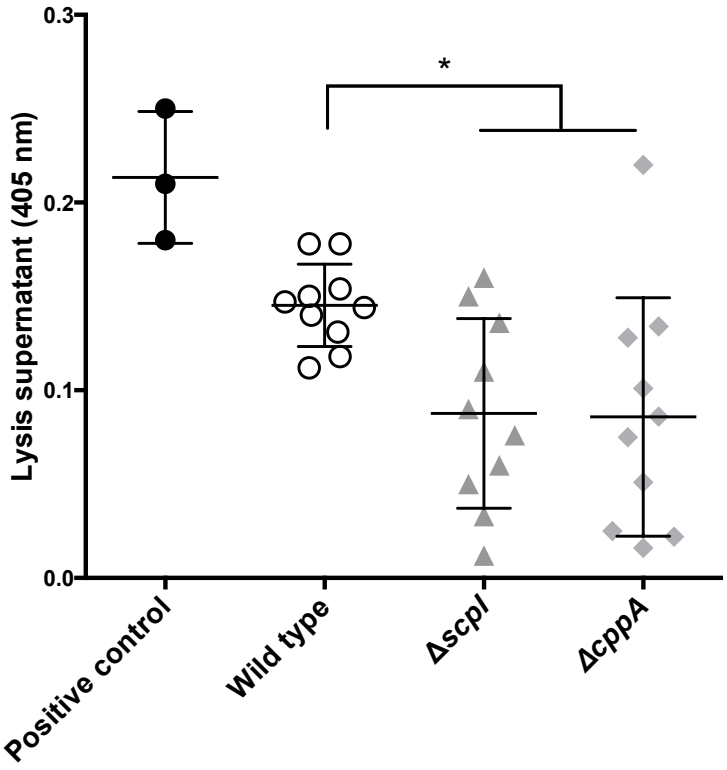

*Figure 14. Haemolytic activity of QMA0248 wild type, ΔscpI and ΔcppA against sheep red blood cells. Positive control (n=3) was completely lysed by adding Triton X-100. (Mean ± SD, n = 10; *p<0.06)*

3.3.2 Knock-out mutants Δ*scpI* and Δ*cppA* did not grow in whole blood

The survival of the wild type QMA0248 in whole blood was consistant with our previous reports. Briefly, the mid-exponential inoculum doubled within the 1 h incubation, whereas the late log inoculum resulted in modest killing (76% survival during 1 h incubation)(Figure 15). In contrast, mid-log inocula of the knockout mutants Δ*scpI* and Δ*cppA* were static or marginaly killed by whole blood (104% and 88% respectively)(Figure 15). Intriguingly, while the susceptibilities of WT and Δ*scpI* mutant during late log phase were similar, Δ*cppA* was more resistant with some growth in whole barramundi blood (survival = 124%, Figure 15).

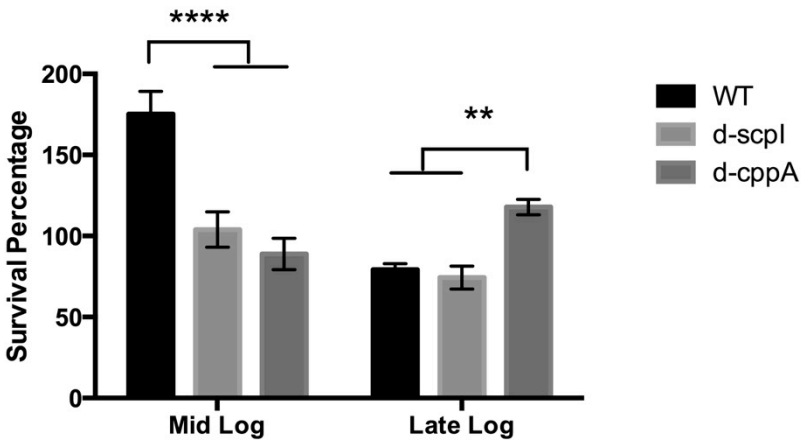

*Figure 15. Survival of QMA0248 wild type, ΔscpI and ΔcppA, at different growth phases in barramundi whole blood. (Mean ± SD, n = 3; **p<0.01; ****p<0.001)*

3.3.3 Knock-out mutans Δ*scpI* and Δ*cppA* are susceptible to host complement

As with previous results (Chan N. Dieu, 2015) the wild type *S. iniae* QMA0248 grew rapidly in serum, more than doubling in cell number during the 30 min incubation, regardless of growth phase of the inoculum, although growth was less rapid in antiserum compared to normal serum (Figure 16 A, B). Growth of the knockout mutants was significantly restricted in heat-inactivated normal serum (HINS) compared to wild type (Figure 16A), and was similar to HINS in HIAS, but not significantly different to the wild type (Figure 16B). When fresh serum was added to HINS as a source of complement and other heat labile plasma proteins, the growth of WT was lower than detected in HINS alone (Figure 16 A, C), but there was no significant difference between WT and either KO mutant. Interesting growth of d-cppA was significantly increased compared to d-scpI. In contrast when FS was added to NIAS growth of both knockouts was severely depleted compared to WT (Figure 16D).

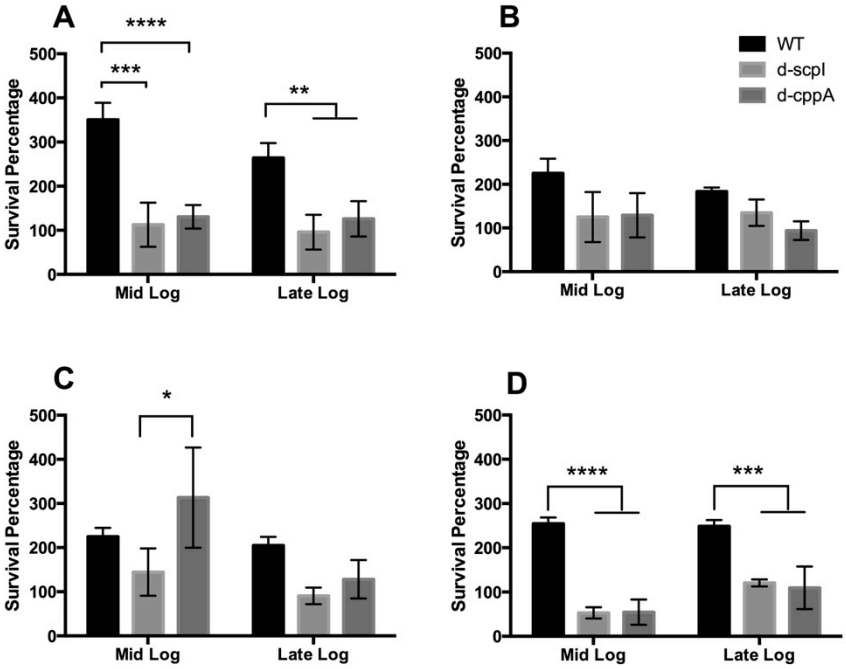

*Figure 16. Growth or survival of QMA0248 wild type, ΔscpI and ΔcppA inoculated at different growth phases into (A) normal serum, (B) anti-QMA0248 serum, (C) normal serum with fresh serum added, and (D) anti-QMA0248 antiserum with fresh serum added. (Mean ± SD, n = 3; * p<0.05; **p<0.01; ***p<0.005; ****p<0.001)*

3.4 Dicussion

The susceptibility of *S. iniae* to host humoral innate immunity over the growth phases investigated by whole blood and serum killing assay reported in our previous paper implicated a trade-off between the expression of capsule and putative complement factor proteases during late log phase (Chan N. Dieu, 2015). Survival in whole blood reflects the susceptibility to the total bactericidal activity of both humoral and cellular components e.g. neutrophils, thrombocytes, macroglobulins, lectins and the complement cascade. In contrast, comparison between heat-inactivated serum and HI serum with fresh serum added as a uniform source of complement is indicative of susceptibility to complement. Therefore the serum resistance of late Log phase inocula (capsule expression reduced) is suggestive of a role for complement proteases in complement resistance, and therefore virulence. Interestingly, a previous study using Δ*scpI* indicated no reduction in virulence in a hybrid striped bass and zebra fish challenge model by IP and IM routes (Locke et al., 2008). These authors speculated on the possible role of other complement proteases to explain the lack of effect of the Δ*scpI* knockout (Locke et al., 2008). In my study I explored

the effect of deletion each of two complement proteases, Δ*scpI* and Δ*cppA* and found them more susceptible compared to the wild type to the host humoral innate immunity *in vitro*. Heat inactivated (HI) normal serum supplemented with fresh serum (FS) is likely to enable both the lectin pathway and the alternative pathway (Mayilyan et al., 2008). While the growth/survival of the Δ*scpI* KO mutant were not substantially affected by addition of FS to HINS, growth of the WT was reduced compared to HINS alone (Figure 16A, C), and the Δ*cppA* mutant, inoculated at mid-log inoculum grew more rapidly in HI normal serum when FS was added (Figure 16C). These results suggest that these proteins are not involved in resistance to complement activated via either the lectin or alternative pathway. Heat inactivated antiserum contains specific antibodies (detected by ELISA) against the WT *S. iniae* QMA0248. Addition of FS to HIAS will mean that the classical complement pathway is also active in addition to the lectin and alternative pathways. In HIAS without FS, growth of WT was restricted compared with HINS, so antibody has a direct role in restricting growth of WT *S. iniae* even in the absence of either cells or complement. I stress that this is inhibition of growth, rather than direct killing as survival was still in excess of 100% of the inoculum, and this may be due to direct blocking of processes vital to nutrient acquisition etc. thereby restricting growth and cell division. The knockout of either *scpI* or *cppA* had no significant effect on growth of *S. iniae* in HIAS (Figure 16B). In HIAS + FS, the growth of the knock-out mutants was significantly restricted compared to the wild type regardless growth phases, with 50% killing evident in mid-log phase (Figure 16D). This is surprising as I have previously shown that these genes are down regulated during high capsule expression in mid log phase relative to late log phase, yet the knockout impacted survival regardless the growth phase. This may reflect the fact that down regulation is not the same as complete deletion and that there is likely to be some basal expression of the proteases during down regulation. In contrast, the knockout mutants will not express the proteases at all and this may not only increase susceptibility to innate immunity, but generally, also have pleiotropic effects because these proteases may have multifactorial roles in growth and survival. It is highly likely that the *scpI* and *cppA* have roles in either modification of the bacterial cell surface, thereby reducing antibody cross reactivity, directly interact with antibody preventing binding or activation of the classical pathway. C3-degrading protease SpeB may also selectively cleave antibodies at the hinge region once they bind to antigens on the surface of *S. pyogenes* (Söderberg et al., 2008), cleave pro-interleukin-1β (Kapur et al., 1993a), hydrolyze H-kininogen

(Herwald et al., 1996), induce histamine release and mast cells degranulation (Watanabe et al., 2002) which enhances *S. pyogenes* infection. It is therefore worth conducting an *in vivo* challenge model with Barramundi *Lates calcarifer* employing these mutants to determine whether there is an effect on virulence in this host-pathogen model. Moreover a dual knock out mutant should be investigated to determine any interaction between these proteases, and amongst the intermediates of complement cascade. Perhaps most importantly, determining any the activity of recombinant *scpI* and *cppA* against a range of complement factors and antibody should be investigated as bacteria can take advantage of the complement-regulatory proteins of the infected host to interfere with the complement system of that host. For example, host complement factor H is a crucial inhibitor of the alternative complement pathway (Müller-Eberhard and Schreiber, 1980). In the absence of specific antibodies, factor H binds to the sialic acid capsule and Streptococcal M protein of the bacteria to protect them from the alternative complement pathway (Brown et al., 1983; Giannakis et al., 2003). The binding of fH to M protein on the Streptococci surface prevents the formation of C3 convertase C3bBb (Jones and Fischetti, 1988), and acts as the cofactor for factor I to degrade C3b as well as stimulates the dissociation of factor B from C3- and C5-convertases (Lars et al., 2002). In addition, factor H can bind to the integrin Mac-1 (CD11b/CD18), which promotes the response activity of neutrophils (DiScipio et al., 1998). Intriguingly, factor H is still retained in the serum heated at 56^0C for 30min (Ram et al., 1998). Therefore the presence of factor H might help our protease knock out mutants survived in heat-inactivated sera (with out complement activities) (Figure 16A, B) and were slightly resistant to the serum killing in HINS+FS (Figure 16C). A protein equivalent to the M-protein of *S. pyogenes*, SiMA has previously been characterized in *S. iniae* (Baiano et al., 2008), and reduces phagocytosis and respiratory burst in fish macrophages. Binding of factor H to *S. pyogenes* was dependent on the hypervariable region of different M proteins, yet this interaction was not sufficient to confer complement resistance on the pathogen (Gustafsson et al., 2013). In *S. pneumoniae*, factor H-binding is inversely correlated with factor B-binding, C3b deposition, and neutrophil clearance, and is closely associated with the capsular serotypes and increased invasion (Hyams et al., 2013). In addition to factor H, others complement-regulatory factors may be recruited by pathogenic bacteria. For example, CD46 plays as a keratinocyte receptor of the bacterial M protein (Okada et al., 1995); C4b-binding protein (C4BP), an inhibitor of both classical and lectin pathways, is also bound by M protein in *S. pyogenes*

(Thern et al., 1995). The binding of M proteins to C4BP as well as factor H cause the increased invasiveness of the pathogen (Blom et al., 2009), but *S. iniae* equivalent of this protein was not effective as a vaccine target in fish (Aviles et al., 2012).

In conclusion, although the single isogenic knock out mutants Δ*scpI* and Δ*cppA* had apparently lower fitness in whole blood and serum than the wild type at both mid- and late-log phases, the interaction between these proteases and others in the complement system is complex and requires further investigation. In order to understand how these proteases are involved in the breaking down the host complement pathway, a complement fixation assay should be conducted. Finally, a rescue of these genes in the knockouts should be performed to demonstrate that the rapid growth in blood and serum can be restored and to confirm their roles in *S. iniae* pathogenesis.

4. Chapter IV. Effect of iron limitation on the coordinate regulation of complement proteases and capsule in *streptococcus iniae*

4.1 Introduction

The process of pathogenesis of *S. iniae* from its environmental niche outside the host through septicaemic dissemination followed by attachment and transit across the microvascular endothelium into the host meninges will necessitate crossing through several different and continuously changing environments to which the pathogen needs to adapt. During all of these phases, resistance to host innate immunity will be critical, therefore the regulation of virulence genes involved in resistance coordinated with requirements for colonization will be required. Whilst the broad range of changes encountered in closed laboratory culture through growth provide a widely inclusive model for study, changes in the host environment that lead to specific gene regulation are also likely to be quite specific. These may include, for example, trace elements, i.e. iron, magnesium, zinc, copper, cobalt that have been demonstrated to play an important role in the regulation of gene expression. During infection, Streptococcal pathogens must acquire the essential elements, in an environment where they are rare or withheld tightly by the host. However some of these essential elements may be harmful in high concentration (eg. Copper and iron) therefore proper regulation of metal homeostasis is required (Higgins, 2001). For example, membrane bound metal transporters MtsABC in *S. pyogenes* in which iron and zinc ion are the two essential ligands (Janulczyk et al., 1999), PsaA in *S. pneumonia* translocates manganese ions (Dintilhac et al., 1997) and is also required for virulence (Berry and Paton, 1996) and a potential vaccine candidate (Talkington et al., 1996). As iron acquisition has a pivotal influence on pathogenesis (Ratledge and Dover, 2000), the regulation of intra- and extra-cellular iron accumulation, and effect of iron limitation on the expression of virulence factors has been widely investigated (Griffiths and McClain, 1988; Smoot et al., 2001b). For example, key virulence factors such as M proteins (Aviles et al., 2013; McIver et al., 1995), cysteine proteases, serine proteases belonging to the same family as scpI (Allard et al., 2006) are up-regulated under iron limitation.

Since expression of the complement proteases and capsule is coordinately regulated over the growth phases (Chan N. Dieu, 2015), and the iron is always withheld tightly by host

high-affinity iron binding proteins such as haemoglobin, lactoferrin and transferrin (Cassat and Skaar, 2013), in this study I aim to investigate the effect of iron limitation on this regulation of *scpI*, *cppA* and capsule at different points of the growth curve.

4.2 Materials and methods

4.2.1 Bacterial strains and culturing

Streptococcus iniae strains QMA0248 and QMA0076 (Aviles et al., 2013) were stored in Todd-Hewitt broth (THB) containing 20% glycerol at -80^{0}C. Isolates were cultured routinely on sheep blood agar plate (Oxoid, Australia) at 28^{0}C for 12-24 h. For broth starter cultures, a single colony was used to inoculate Todd-Hewitt broth and incubated at 28^{0}C with gentle agitation for 12-18 h. For growth curves, 30 µl aliquots taken from overnight broth cultures, measured the optical density (OD) at 600 nm and adjusted to 1.0, were used to inoculate 1470 µl fresh THB in 24 well tissue culture plates. Growth was recorded by measuring optical density 600 nm over time using a POLARstar Omega Microplate Reader (BMG Labtech, Melbourne). Mid-exponential, late exponential, and stationary phases were determined from at least three replicate experiments with each plate incorporating un-inoculated negative control wells.

4.2.2 Determination of gene expression

In order to determine the effect of iron limitation on the coordinate regulation of capsule with the complement proteases, the gene encoding a putative transferase *cpsE* in the capsular operon (Lowe et al., 2007) was chosen for study together with *scpI* and *cppA* under the Fe-limited conditions. For normalization of gene expression, three housekeeping genes *gyrA*, *glnA*, and *sdhA* were also included as previously described.

S. iniae strains QMA0248 and QMA0076 (Aviles et al., 2013) were cultured in Todd-Hewitt broth (THB) as described in previous chapter. For iron limitation, THB was supplemented with 2,2-dipyridyl (DP) to a final concentration of 1mM. Each strain was cultured in 24-well plate with three biological replicates in THB and THB+DP. The sample points were mid-exponential phase, late exponential phase, and stationary phase. RNA extraction and real-time PCR procedures followed the protocol described in our previous paper (Chan N. Dieu, 2015). Briefly, at each sample point, 1ml subsamples were immediately pelleted at

10,000 x g for 2 min at room temperature and re-suspended in 1ml of TRIsure ^{TM} (Bioline US Inc) and transferred to a 2 ml screw-capped tube containing MagNA Lyser Green Beads (Roche Diagnostics, Germany) then homogenized at 7,000 rpm for 40s in a Roche MagnaLyser and immediately chilled on ice. The supernatant was transferred into the new tube and 200µl chloroform was added. The lysate was shaken vigorously for 15s, incubated at room temperature for 2 min then centrifuged at 12,000 x g for 15 min at 4^0C. The aqueous phase was transferred carefully to a new tube, and the RNA was precipitated by adding 500 µl of isopropyl alcohol followed by centrifugation at 10,000 x g for 10 min at 4^0C. The RNA pellet was washed with 1ml of ethanol 75% and air-dried for 3h. 30 µl of DNase- and RNase-free water was added to re-suspend the pellet followed by storing at -80^0C for further study. RNA was quantified fluorimetrically using a Qubit fluorimeter (Life Technologies, Melbourne) and 20ng per sample was used for cDNA synthesis with the QuantiTect® Reverse Transcription Kit (Qiagen®) in accordance with the manufacturer's instructions.

All real-time PCR experiments were performed in 384 well plates with reagents dispensed using an epMotion 5075 Robot (Eppendorf, Hamburg) and thermal cycling was performed with the ABI ViiA7 using SYBRGreen Master Mix (Applied Biosystems). The primers and input cDNA concentrations were optimized at 200nM and 1ng respectively per reaction to yield ~100% efficiency. In each reaction there were 0.2 µl of Forward primer, 0.2 µl of Reverse primer (both from 10 µM stock solutions), 5 µl of SYBRGreen Master Mix, 2 µl of cDNA 0.5 ng/µl, and 2.6 µl of water. Cycling parameters were: 95 ^{0}C for 10 min followed by 40 cycles of 95 ^{0}C for 15 s and 62 ^{0}C for 1 min, then a final melt curve at 95^0C for 15s, 60^0C for 1min and 95 ^{0}C for 15 s. All temperature cycling was performed with acceleration at 1.6 ^{0}C/s.

4.2.3 Data analysis

The amplification efficiency was accounted for during normalization of relative gene expression differences between the target group and the control group. The normalization factor was calculated based on the geometric mean of the relative quantities valued for three reference genes using REST (Pfaffl et al., 2002; Vandesompele et al., 2002). Ct data were analysed with REST based on the Pair Wise Fixed Reallocation Randomisation Test (Pfaffl et al., 2002), in which the normalized relative quantities were calculated based on

the ratio of the group means for target gene against the normalized reference genes. The result represented the up- or down-regulation of the target groups (i.e. each of the three growth phases under iron limitation condition) compared to their respective control group (i.e. each of the three growth phases under iron normal condition). Bacterial growth curves were analyzed by non-linear regression based on the Gompertz equation (Mytilinaios et al., 2012).

4.3 Results

4.3.1 Bacterial growth and sampling points

In order to investigate the coordinate regulation of *cpsE* and the putative complement proteases under the iron limitation condition, the growth curves were constructed for each of the bacterial isolates. From the curves potential transition points were identified for comparison of gene expression between iron-normal and –limited conditions. Transition from log to stationary phase was identified in each curve and samples taken during mid-log, late-log and stationary phase under iron-normal condition were as the references for their corresponding sampling points under iron-limited condition (Figure 17). The incubation time points when samples were taken are presented in Table 1.

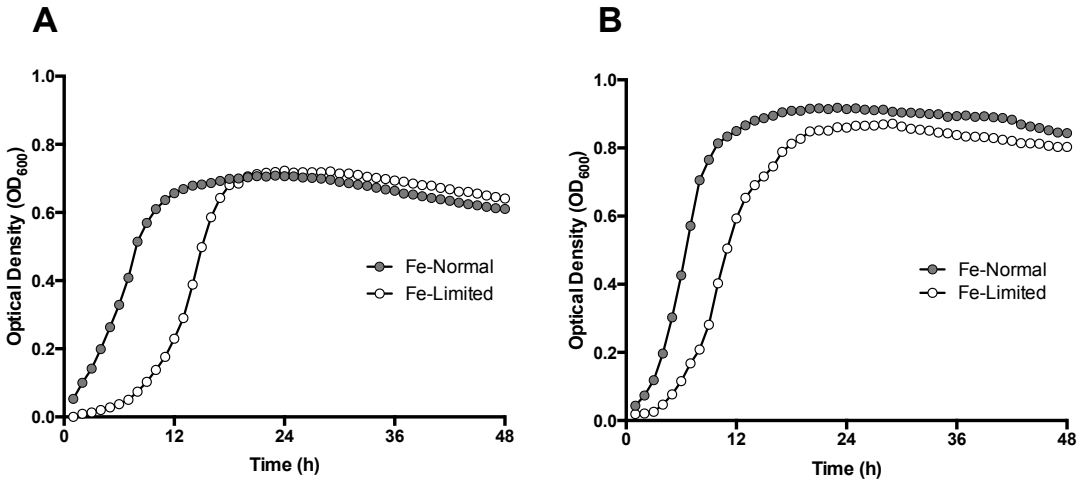

Figure 17. Growth of S. iniae strains (A) QMA0076 and (B) QMA0248 grown under Fe-normal and Fe-limited conditions

Table 1. The sampling time points corresponding to three growth phases under Fe –normal and –limited conditions

Strain	Condition	Time cells harvested		
		Mid-exponential	**Late-exponential**	**Stationary**
QMA0076	Fe-normal	6 h 00 min	9 h 00 min	24 h 00 min
	Fe-limited	13 h 00 min	21 h 00 min	27 h 00 min
QMA0248	Fe-normal	5 h 30 min	8 h 50 min	24 h 00 min
	Fe-limited	11 h 00 min	19 h 00 min	27 h 00 min

4.3.2 Effect of iron limitation on the coordinate regulation of complement proteases and capsule

The expression of the three genes of interest under iron limitation relative to normal THB culture was consistent between strains (Figure 18). During mid log phase, expression of cps was significantly up regulated under iron limitation compared to normal THB culture at the same point (Figure 18). In contrast *scpI* was significantly down regulated compared to normal THB culture. Expression of *cppA* was not significantly affected by iron limitation during mid- or late- exponential phase in either strain tested (Figure 18), but was significantly up regulated during stationary phase under iron limitation compared to growth in THB (Figure 18). In contrast, *cpsE* was significantly down regulated under iron limitation compared with THB during stationary phase (Figure 18).

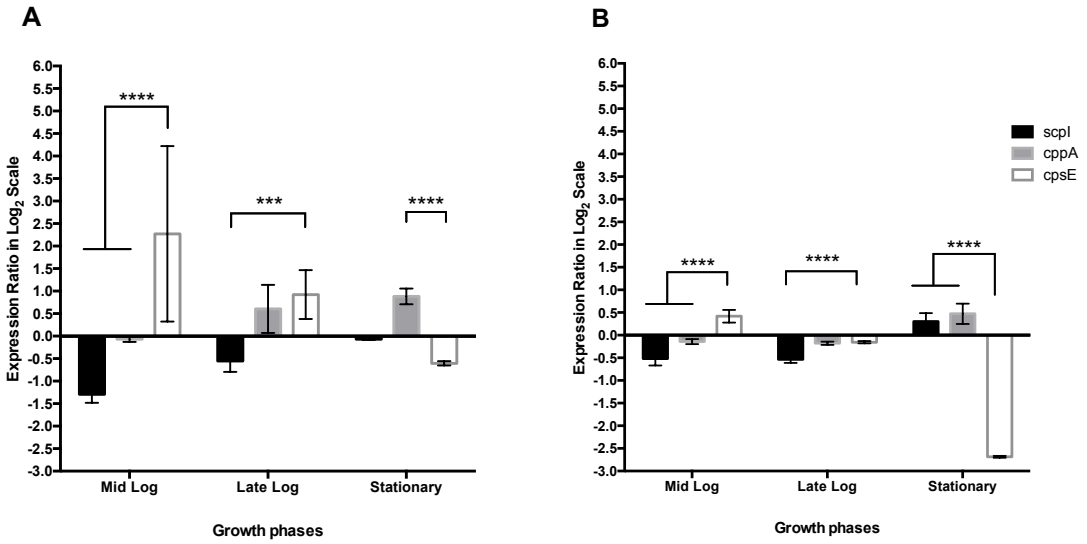

Figure 18. Expression (normalized against three reference genes) of scpI, cppA, and cpsE at three growth phases under Fe-limited condition relative to Fe-normal condition. (A) S. iniae QMA0076, (B) S. iniae QMA0248 (Mean ± SD, n = 9)

4.4 Discussion

I investigated the effect of iron limitation as iron is tightly withheld by host high-affinity iron binding proteins such as haemoglobin, lactoferrin and transferrin (Cassat and Skaar, 2013). By comparing gene expression at each growth phase under iron limitation with corresponding expression under normal growth in THB relative to normalized reference genes I was able to confirm our previous observation that CPS and *scpI* (and possibly *cppA*) are coordinately regulated (Chan N. Dieu, 2015). Moreover, one of the potential inducers of regulation in the host may be the lack of available iron. Free iron in the host is extremely limited (Litwin and Calderwood, 1993) and bacteria must employ diverse mechanisms in order to free iron, or utilize the host iron-binding compounds directly (Litwin and Calderwood, 1993; Neilands, 1981, 1982). Competitive growth and survival inside the host requires adaptive responses to iron stress and a number of iron acquisition strategies, but is also a signal for the host phase of the lifecycle of pathogenic or commensal organisms. The depression of *cpsE* and consequent capsule depression by *S. iniae* under iron stress may be such an adaptive response. In the blood, capsulated *S. iniae* causes septicaemia, but capsule-deficient mutants preferentially enter CNS instead of remaining in blood stream resulting in strikingly different pathology (Millard et al., 2012). It may be that depleted iron reserves are a signal that *S. iniae* has entered the host resulting in

immediate up-regulation of the capsule in capsulated strains, allowing rapid dissemination through the blood as observed during pathology (usually 24-48 h in warm water fish). Subsequently, the capsule is down regulated facilitating attachment, but leaving the bacteria potentially exposed to complement. This exposure is ameliorated by relative up-regulation complement proteases. Further work is required to determine what factors in the host may regulate this later switch.

Nutrient stress in addition to iron stress still occurs when the bacteria life span enters the stationary phase (Bacun-Druzina et al., 2011). Therefore it is highly likely that multiple interacting factors effect regulation of these systems during growth phase; for example elevated CO_2, superoxide level, oxygen level, and other metal ions such as zinc or manganese (Kreikemeyer et al., 2003). Moreover, there is likely to adaptation to iron limited conditions over time as cells activate additional iron acquisition, transport and storage systems to better compete with the host for available resources. For example, MtsABC transports Fe^{2+} and Mn^{2+} in both *S. pyogenes* and *S. agalactiae* (Ge et al., 2009), iron-binding protein Dpr that required oxygen tolerance in *S. mutants* (Yamamoto et al., 2004). In addition, when the *Streptococcus gordonii* grow in an iron-depleted environment, they increase acquisition of Mn^{2+} (Jakubovics and Jenkinson, 2001; Jakubovics et al., 2000), and engage the oxidative stress responses (Bsat et al., 1998; Storz and Imlay, 1999). Oxidative stress from endogenous as well as extracellular sources requires an effective defence mechanism. $O_2\bullet^-$ and H_2O_2 inside the bacterial cell can cause the oxidative damage to the exposed iron sulfur clusters (Imlay, 2008), damage to bacterial proteins, DNA, and lipids (Imlay, 2003). Much of this damage is due to iron catalysed Fenton chemistry and therefore iron and oxidative stress are tightly linked. However the ability of H_2O_2 to depress the iron uptake system via, for example the PerR regulon, is dependent on the iron/manganese ratio within the cell (Faulkner and Helmann, 2011), i.e. in the abundant presence of Mn^{2+}, PerR permits uptake iron despite accumulation of H_2O_2 (Chen et al., 1995; Fuangthong et al., 2002) because manganese acts as a quencher of hydrogen peroxide, helping to protect the cells from oxidative damage (Stadtman et al., 1990). Also tightly inter-linked with manganese, zinc is the second most abundant metal in the host and has the essential role in the immune system (Fischer Walker and Black, 2004; Rink and Gabriel, 2001). Zinc is released in excess at the mucosal surfaces and can be toxic to bacteria (Silver, 1996). Zinc is also known as inducer of the adhesion of myelomonocytic cells to the endothelium, which is critical to the recruitment of leukocytes

to the infected site (Maret, 2001). Zinc deficiency leads to decreased chemotaxis of neutrophils (Maret, 2001). In *S. pneumoniae*, Zn^{2+} appears to compete with Mn^{2+} to bind with PsaA, an ATP-binding transporter (Berry and Paton, 1996; McDevitt et al., 2011); moreover, binding of Zn^{2+} to PsaA may be irreversible (McDevitt et al., 2011). The high ratio of zinc/manganese leads to restricted growth of *S. pneumonia*, and increased susceptibility to oxidative stress and leukocyte killing (McDevitt et al., 2011). Consequently, as the availability of Zn^{2+} and Mn^{2+} are intrinsically linked to iron and oxidative stress, the factors that regulate complement proteases and capsule need to be further investigated under other conditions such as elevated H_2O_2, and variant iron/manganese and zinc ratios.

In conclusion, I have demonstrated an effect of iron limitation on the coordinate regulation of complement proteases and capsule expression in *S. iniae*. However, this regulation appeared to be affected by multiple factors that need to be further investigated.

5. Chapter V. Discussion

5.1 Bacterial strategies to counter the host complement system

Bacterial pathogens causing septicaemia and meningitis in farm fish, and potentially in humans, are extremely diverse, including Gram negative pathogens such as *Aeromonas hydrophila*, *Edwardsiella tarda*, *Erysipelothrix rhusiopathiae*, *Photobacterium damselae*, *Vibrio* spp. to the Gram positives which mainly comprise members of the Streptococci (Austin and Austin, 2012). Although different pathogens possess variable pathogenesis mechanisms, they share similar basic invasion steps to successfully enter the host, including colonization, establishment, compromise of host defences, and host cell and tissue damage (Telser, 2002). Pathogens have recruited a variety of strategies to overcome the non-phagocytic and phagocytic defence mechanisms including modification of membrane receptors to delay host immune recognition, bacterial membrane alteration to adapt to antimicrobial peptides, prevention of opsonisation, capsule formation, toxin-mediated necrosis induction, etc. (Hornef et al., 2002). Successful escape from internalization and/or destruction by phagocytes is essential to systemic spread of bacteria during blood infection (Hornef et al., 2002), and bacteria have consequently adapted a diverse array of mechanisms for phagocyte evasion. For example, Streptococcal streptolysin depresses neutrophil chemotaxis during the early stage of *S. pyogenes* infection (Lin et al., 2009). Furthermore, physical prevention of phagocytosis or disguise from phagocyte pattern recognition receptors by, for example, polysaccharide capsule, M protein and fimbriae as reported in GAS (Urban et al., 2006). In order to escape innate humoral factors, the complement proteases directly inactivate the complement system by disrupting the essential intermediate components, C5a and C3a, the most potent anaphylatoxins that induce the pro-inflammatory response (Yancey and Lazarova, 2008). These compelement protein subunits have a pleiotropic effect on the chemoattraction of neutrophils, monocytes, macrophages, and T and B lymphocytes (Becker, 1972; El-Naggar et al., 1980; Snyderman et al., 1975). The disruption of these two C3 and C5 may block complement deposition since they are the essential components of C3 convertase and C5 convertase complexes, which are integral in the down stream completion of all currently understood complement pathways (Yancey and Lazarova, 2008). The C5a degrading peptidase, Scp (known as ScpA in *S. pyogenes*, ScpB in *S. agalactiae*), is able to cleave C5a slowing the influx of polymorphonuclear leukocytes through failure of

chemotactic signaling and neutrophil attraction to the infection site (Cleary et al., 2004). CppA, the C3 degrading protease, was first characterized in *S. pneumoniae* and completely degrades the α-chain of human C3 protein (Hostetter et al., 2004). The disruption of C3 can stop the formation of both C3 and C5 convertase complexes, with consequent failure of bacterial opsonisation (Yancey and Lazarova, 2008).

In spite of the substantial gene loss encountered in marine Streptococci through the reductive evolution in comparison to their terrestrial counterparts, putative homologues of the proteins are present in the genomes of *S. iniae* (Baiano and Barnes, 2009), and are of interest as potentially important virulence factors and therefore formed the basis of investigation in this thesis. First, I established a strong informatics basis for their putative roles as secreted complement-degrading proteases: I identified signal peptides and cleavage sites using Signal P4.1 (Nielsen et al., 1999b) and ProParam confirming likely direction of ScpI to the secretion pathway: ScpI possesses a putative twin arginine at residues 32 and 33, while it is at residue 31,32 in ScpA (Chen and Cleary, 1990). Subsequently, the putative proteolytic active site and metal binding domains were identified with Phyre2 (Sternberg and Kelley, 2009). There is also a S8 peptidase domain highly homologous with ScpA along with the active cleavage site triad at residues Asp114, His181 and Ser501, while those residues in ScpA and ScpB are Asp130, His193 and Ser512 or Ser509 (Chen and Cleary, 1990; Siezen et al., 1991). *Streptococcus iniae* CppA is highly homologous to the CppA N-terminal domain in *S. pyogenes* and C3-degrading protease in *S. dysgalactiae*. However there was neither detectable signal peptide nor predictable transmembrane helices, or beta barrel or membrane anchor. Teleost fish possess a potent complement system and functional complement protein homologues (Boshra et al., 2006; Holland and Lambris, 2002). The cytolysis and opsonisation for later phagocytis have been confirmed in a wide range of fish (Byon et al., 2006; Gonzalez et al., 2007; Nonaka et al., 1985; Sakai, 1984). However, the fish complement proteins do possess many structural isoforms which may be transcribed from more than one gene (Holland and Lambris, 2002). Indeed, the genes encoding for C3 in fish are polymorphic and may need multiple mechanisms to activate (Jensen and Koch, 1991; Nonaka et al., 1985). Therefore, while ScpI is a potential candidate for C5a cleaving protease in fish, CppA requires some further investigation, particularly in light of its lack of tat pathway secretion or signal peptide and consequently its predicted cytoplasmic location.

Fish pathogens may target the complement system in at other points in the pathway. For example, in *Neisseria meningitidis*, NalP protease can cleave both C3 and C4 (Tordello et al., 2014); the PgtE protease of *Salmonella enterica* is able to cleave C3b, C4b, and C5 to facilitate the serum resistance (Ramu et al., 2007); the Alp1 protease in *Aspergillus fumigatus* is an effective protease that can cleave a variety of complement proteins, i.e. C1q, C3, C4, C5, and also IgG (Behnsen et al., 2010). This multiple function property can also be found in Group A Streptococci SpeB protein. Mature SpeB can cleave the C3 component and prevent the formation of the C5 convertase complex (Kuo et al., 2008; Terao et al., 2008), lyse Properdin leading to a labile complex subsequently been inactivated (Tsao et al., 2006). Furthermore, isogenic Δ*speB* mutants cause lower mortality and tissue damage than the wild type (Lukomski et al., 1997). SpeB cysteine protease is also involved in the damage of mitochondrial of polymorphonuclear cells that help *S. pyogenes* escape from phagocyte clearance at the early infectious stage (Chiang-Ni et al., 2006). SpeB may also selectively cleave antibodies at the hinge region once they bind to antigens on the surface of *S. pyogenes* (Söderberg et al., 2008). Furthermore, this protease can also hydrolyse and destroy chemokines, which are secreted by inflamed epithelial cells, and are responsible for signalling and antibacterial activity (Egesten et al., 2009). SpeB can also induce inflammation by cleaving pro-interleukin-1β into active form interleukin-1β (Kapur et al., 1993a), hydrolysing H-kininogen into bradykinin (Herwald et al., 1996), inducing histamine release and mast cells degranulation (Watanabe et al., 2002), which enhances *S. pyogenes* infection. It is likely SpeB is an important part of the multi-target invasion strategy of S*. pyogenes* as it effectively degrades innate immune barriers including fibrin/fibrinogen in blood coagulation (Elliott, 1945; Matsuka et al., 1999), antigen-bound plasmin (Svensson et al., 2002), extracellular matrix proteins vitronectin and fibronectin (Kapur et al., 1993b); and indirectly participates in tissue degradation and apoptosis (Burns et al., 1996; Tamura et al., 2004). The multiple roles of SpeB in the virulence of terrestrial Streptococcal strains merit further investigation of additional roles for *scpI* in *S. iniae* and searching the genome for alternative potential C3-degrading protease.

5.2 Relationship between growth phase and gene expression

During the bacterial life span, a typical growth curve has four stages: lag phase, exponential growth phase, stationary phase, and finally, death phase (Bell, 2000). There are few studies of lag phase, but it is assumed to be an adaptation phase before entering a new environment that allows bacterial self-reparation e.g. macromolecular damage during previous stationary phase or dormancy (Dukan and Nyström, 1998; Madigan, 2015). Exponential phase is when the cells divide with an exponential rate and requires a large amount of nutrient including carbon, nitrogen, phosphate, and trace elements such as iron (Madigan, 2015). During this expansion phase synthesize a large amount of DNA, along with proteins and their regulators are synthesized (Madigan, 2015). As a variety of stresses such as nutrient, density, toxin, waste, etc. accumulate, bacteria enter stationary phase where the rate of accumulation of new cells is equal to the death rate (Madigan, 2015). Indeed, in the natural environment, particularly in the host, where the nutrient such as trace elements are usually withheld as a host defence strategy, the bacteria are often found in starvation conditions until infection progresses sufficiently for bacterial toxins to release nutrients from degraded or necrotic tissue (Bacun-Druzina et al., 2011). Therefore pathogenic bacteria have to undergo a number of morphological and physiological changes as an adaptive response on host entry (Zinser and Kolter, 2004). The effect of growth phase on gene expression has been studied for decades, and it is one of the critical mediators of environment-dependent change of bacterial protein expression (McIver and Scott, 1997; Unnikrishnan et al., 1999). In order to adapt to the known or unknown new environment, the bacteria expresses some core proteins that they continue to produce from the previous environment, along with a range of adaptive proteins including virulence factors that help them to survive in the new niche or host (Smoot et al., 2001a; Unnikrishnan et al., 1999). The array of genes involved in mediating and executing the adaptive strategies are expressed dependent on stage of bacterial growth and on conditions in the environment around them, so called growth phase regulation (Barnett et al., 2007; Hornef et al., 2002). For example, the expression of virulence gene regulator *mga* in *S. pyogenes* is maximal in the exponential phase and is shut down in the stationary phase, along with Mga-regulated genes *scpA* and *emm*, in order to save energy under starvation condition (McIver and Scott, 1997). Moreover, the expression of toxin genes is delayed to rescue the bacteria in stressful conditions. For example, Streptococcal pyrogenic exotoxin A *speA* from *S. pyogenes* is a phage-encoded toxin (Weeks and

Ferretti, 1986) and its expression is delayed until the late log phase; Streptococcal pyrogenic exotoxin B *speB* is only expressed under starvation or stressful conditions (Chaussee et al., 1997); no detectable SpeB is expressed in antibiotic-free or high nutrient medium as detected by Northern blot hybridization (LeBlanc et al., 1986). With the clear evidence of growth phase regulation of virulence genes in Streptococcal pathogens, it is a reasonable starting point for investigating coordinate regulation of complement proteases with capsule expression.

5.3 The coordinate regulation of complement proteases and capsule

In chapter two, I demonstrated the coordinate regulation of complement protease expression with CpsE expression in late exponential phase. In Streptococci, polysaccharide capsule is considered the major virulence factor which varies depending on serotype (Lowe et al., 2007). The variation of capsule from group B *Streptococcus* helps to reduce the C3b opsonisation (Martins et al., 2010). In *S. iniae*, the expression of capsule has been demonstrated necessary for infection (Miller and Neely, 2005) in a zebrafish model. Interestingly, the capsule deficient variants of *S. iniae* preferably entered the spinal column of barramundi, but do not cause the characteristic septicaemia and meningitis (Millard et al., 2012). In addition, capsule is unnecessary for the colonization by *S. pneumoniae* of bronchoepithelial cells (Magee and Yother, 2001; Morona et al., 2004). Moreover, an uncapsulated isolate of *S. pneumonia* had higher invasive capacity than the capsulated isolate (Adamou et al., 1998), suggesting the possibility the *cps* expression may impede attachment to an invasion of cells. Therefore, the coordination of *cps* expression with complement proteases *scpI* and *cppA* may be a sound pathogenesis strategy to maintain complement resistance during attachment to host cells for subsequent tissue invasion. I chose *cpsE* as a gene in the capsular operon immediately downstream of the regulatory complex of cpsABCD, and identified previously as a possible priming glycosyl transferase required for cps expression (Lowe et al., 2007). Gene expressions were determined at each growth phase, and the coordinate regulation of *scpI* and *cppA* with *cpsE* occurred in the late exponential phase. In all three growth-phases, the expression of *scpI* was more dramatically changed than the *cppA*. The susceptibility of *S. iniae* wild type isolates over the growth phases was investigated by whole blood bactericidal assay and indicated higher susceptibility of *S. iniae* to whole blood during late log phase, when capsule is down regulated. This result was consistent with the previous

study that a capsule-deficient isolate was susceptible to whole blood (Locke et al., 2007; Lowe et al., 2007). The results are also consistent with my hypothesis that capsule must be down-regulated to allow adherence which may be required for progression of the disease from the blood to the CNS. This was further corroborated by the plastic adherence assay in which the late log inoculum was the most adherent; indeed, capsule has been reported to impede adherence (Magee and Yother, 2001; St Geme and Falkow, 1991).

To tease apart the respective roles of neutrophils and plasma factors such as complement in the inhibition of growth and therefore potential roles for ScpI and CppA proteases. I challenged inocula in each of the three growth phases in a serum killing assay with heat-inactivated (HI) serum supplemented with or without specific antibodies and fresh serum as a source of complement proteins and other heat labile plasma proteins. The resistance to serum killing generally declined over the growth phase, with the exception of resistance to HI specific anti-serum supplemented with fresh serum where the late log inoculum was resistant/survived at a similar level to the mid log inoculum. Surprisingly, the general resistance/survival of these two inocula in the HI specific anti-serum with added fresh serum (HIAS+FS) as a source of complement significantly increased compared to the HI specific anti-serum alone. While the HI normal serum supplemented with fresh serum (HINS+FS) was established as a model where the lectin and alternative pathways are active, the HI specific anti-serum with added fresh serum should also have active classical pathway. The higher survival of late log inoculum in the HIAS+FS may reveal the involvement of *scpI* and *cppA* in resistance to or subversion of the classical pathway, as well as their protective role when *cpsE* is down regulated. However, taken together with the results from whole blood killing assay, the up-regulation of the complement proteases in late log phase did not fully protect the bacteria. The down regulation of *cpsE* resulting in the ineffective capsule (Doran et al., 2005; Lowe et al., 2007) can facilitate the successful adherence (St Geme and Falkow, 1991) in the late log phase. However, the complement degrading mechanism is not the only strategy in this circumstance. The evidence is the late log phase inoculum was still significantly killed (50%) in the whole blood, where neutrophils are likely the major means of bacterial neutralisation. In order to clarify the role of *scpI* and *cppA* in *S. iniae* virulence, isogenic knock out mutants of these genes were prepared and reveal their multiple functions in growth and survival.

5.4 The pleiotropic effect of complement proteases

In chapter three, I explored the phenotype of knock out (KO) mutant Δ*scpI* and Δ*cppA* and found them more susceptible compared to the wild type to host innate immunity *in vitro* regardless the growth phases. In a previous study virulence of Δ*scpI* was investigated in a hybrid stripped bass challenge model and there was no attenuation (Locke et al., 2008). This IP- and IM-injection route might ignore the role of *scpI* in the early stage of infection from the environment. Furthermore, the inactivation of this gene does not always lead to the general reduction in virulence *in vivo*. In a mouse challenge model, Δ*scpA* of *S. pyogenes* was no different to wild type in the incidence of throat colonization, however there was a decrease of incidence of pneumonia caused by Δ*scpA* (Husmann et al., 1997). In Streptococcal species, the complement proteases appeared to be multi-functional in pathogenesis. Besides the ability to cleave and inactivate C5a, Scp can act as an adhesin to Fibronectin III due to the induced conformational change of Fn and enhance the invasion of non-phagocytic cells (Beckmann et al., 2002; Hull et al., 2008). The presence of recombinant ScpA peptidase from *S. pyogenes* delayed the recruitment of polymorphonuclear leukocytes in a mouse IP challenge model. However, there was no difference in virulence between Δ*scpA* and wild type by IP injection *in vivo* (O'Connor and Cleary, 1987). There is evidence of delay of polymorphonuclear leukocyte recruitment to the infection site and chemokine impairment by ScpA and ScpC in GAS (Belotserkovsky et al., 2006) and in GBS (Bohnsack et al., 1993). Although knockout mutants resulted in no effect on mortality in a mouse model (Belotserkovsky et al., 2006; Ji et al., 1996), Δ*scpA* and wild-type followed different paths for dissemination within the infected mice, i.e. Δ*scpA* translocated into the lymph nodes, while the wild type preferentially occurred in the spleen (Ji et al., 1996).

The C3-degrading protease SpeB in GAS can interact with diverse molecules to block immune functions e.g. cleave antibodies (Söderberg et al., 2008), destroy chemokines (Egesten et al., 2009), cleave pro-interleukin-1β into active form interleukin-1β (Kapur et al., 1993a), hydrolyze H-kininogen into bradykinin (Herwald et al., 1996) which enhances GAS infection. In chapter three, this pleiotropic effect may be evident via the phenotypes of the protease knock out mutants when they were exposed to whole blood and sera: they were not only more susceptible compared to the wild type, but also had restricted growth rates. I also observed these complement proteases were not involved directly in resistance

to either the lectin or the alternative complement pathways. However, with the addition of fresh serum to heat-inactivated specific antiserum to activate the classical pathway, I observed the mid-log inoculum of both mutants was significantly killed rather than the late-log inoculum. This result may reflect the fact that there is a basal expression of complement proteases during down regulation in the mid-log phase, but the knockout of the protease expression in the mutants apparently increases their susceptibility to blood killing and also impacts their growth. It is highly likely that ScpI and/or CppA either contribute in the modification of the bacterial cell surface to reduce the antibody cross-reactivity, or interact with antibody preventing binding to activate the classical pathway. For example, polyclonal antibody in rabbit anti-ScpB serum did not function as an opsonin to facilitate the association of macrophage and GBS, however it enhanced the killing activity of the macrophage by promoting the anti-Cps antibody (Cheng et al., 2001). Interestingly, the induced specific antibody which was directly against ScpB was not only opsonic but also reduced the colonization of GBS (Cheng et al., 2001) and inhibited the invasion of GBS into the epithelial cells (Cheng et al., 2002). It is likely that the conjugation ScpB-Cps induces a strong response of functional antibody and enhances the bactericidal activity of macrophage. Therefore, the conjugation ScpB-Cps sounds promising for vaccine production (Cheng et al., 2001).

Bacteria can take advantage of host complement regulatory factors to help them interfere with these pathways. For example, in our serum-killing assay, the survival and slight resistance of the knock out mutants in HINS+FS (with complement activity) may be due to factor H activity. Factor H, a co-factor for factor I, is an essential regulator of the alternative pathway (Müller-Eberhard and Schreiber, 1980). Factor H can bind to the Streptococcal M protein to protect them from the alternative pathway (Giannakis et al., 2003). This binding prevents the formation of C3 convertase (Jones and Fischetti, 1988) as well as dissociates factor B from C3- and C5-convertases (Lars et al., 2002). Furthermore, factor H can bind to Mac-1 which is the integrin promoting neutrophil response (DiScipio et al., 1998). The remaining factor H in the heat-inactivated sera might help the KO mutants escape from the neutrophil-mediated killing. Therefore, the activity of recombinant ScpI and CppA should be investigated against a wide range of complement regulators and antibodies. Moreover, a dual knock out should be studied to determine the interaction between these two proteases as well as amongst the intermediates of the complement cascade.

5.5 The effect of iron limitation on complement proteases and capsule expression

During bacterial growth, the environmental conditions change substantially over the growth phases, as toxins accumulate and nutrients are converted and depleted (Madigan, 2015). The diverse environmental effects encountered through growth phase act upon a wide range of regulatory mechanisms within the cells and make growth phase an excellent set of conditions for investigating coordinate gene regulation. For example, the membrane lipid composition and the virulence gene RpoS/RpoH expression in Salmonella Enteritidis had coordinately regulated in order to adapt to lethal heat and lactic acid stress (Yang et al., 2014); in *Staphylococcus aureus*, O_2 and NO act as an environment cues that induce the regulated expression of variety of virulence genes including T3SS, HlyE, LukD, TSST1 (Green et al., 2014); the dynamic expression of operon *flhDC* which is responsible for flagella in *Salmonella enterica* is growth phase dependent, and is regulated by either LrhA or RcsB in different stages of growth (Mouslim and Hughes, 2014). Consequently, I was able to document inverse coordinate regulation of *scpI* and *cppA* with *cpsE* in the capsular operon. However, the key environmental triggers that drive this regulation in the host during colonization remain to be elucidated. In order to adapt to and escape from the host defence, diverse mechanisms are recruited and these mechanisms require availability of essential nutrients such as iron (Bacun-Druzina et al., 2011). Therefore, limited nutrient conditions act as a signal for launching an adaptive mechanism to this environment (Hornef et al., 2002). Therefore I investigated the expression of *scpI* and *cppA* under iron limitation compared to standard culture media, since the availability of iron is extremely limited during infection due to tight withholding by the host high-affinity iron binding proteins (Cassat and Skaar, 2013). Competitive survival in the host requires the invading pathogen to be adaptively responsive to iron stress. I found that *cpsE*, consequently capsule, is down regulated under iron stress and this may be an adaptive response. The depletion of iron may serve as a signal for immediate up-regulation of the capsule allowing rapid dissemination in the blood stream. The down regulation of capsule in the next growth phase followed by the relative up regulation of complement proteases may enhance the adherence whilst maintaining some protection against host innate immune factors. However which factor triggers this depression is still unknown. The limitation of iron is also linked with Mn^{2+} acquisition (Jakubovics and Jenkinson, 2001), the oxidative

stress responses (Bsat et al., 1998; Storz and Imlay, 1999), and the availability of Zn^{2+} (McDevitt et al., 2011). Furthermore, even in rich culture media, nutrient stress still occurs when the bacteria transform to the next phase in their lifecycle (Bacun-Druzina et al., 2011) and a large number of virulence factors involved in the interaction between host and pathogen is expressed under the control of transcriptional regulators that respond to these kinds of environmental signals (Kreikemeyer et al., 2003).

Two-component signal transduction is a frequently employed mechanism in GAS that links host-environment signals with the bacterial adaptive response, i.e. the transcriptional regulator is triggered by a phosphoryl group pre-stimulated by a sensor histidine kinase as the response to the host signal (Hoch, 2000). Furthermore, this interaction is also controlled by multigene regulons such as the Mga regulon in GAS (Caparon and Scott, 1987). In GAS, *scpA* is regulated by the Mga regulon, which is a growth-phase dependent regulator that maximizes its expression in the exponential phase (Kreikemeyer et al., 2003), and is sensitive to the increase of carbon dioxide, iron starvation and increased temperature (Caparon et al., 1992; Kreikemeyer et al., 2003; McIver et al., 1995). Mga regulates a cluster of virulence genes being responsible for entry, colonization and immune invasion including *emm, scpA, sof, rofA, speB* etc. and *mga* itself (Mühldorfer and Schäfer, 2001). The homologous regulator to Mga in GAS has been found in *S. iniae* called Mgx (Baiano et al., 2008), which is 20-fold increased in expression in the exponential phase under iron limited condition (Aviles et al., 2013). Mgx is the regulator of SiMA, the M-like protein found in *S. iniae*, and correlatively expressed with SiMA in the exponential growth phase when iron was limited (Aviles et al., 2013). Interestingly, the adding of fresh serum which contained labile humoral factors such as complement proteins and fibrinogen reduced the phagocytosis and oxidative burst of barramundi leucocytes (Aviles et al., 2013). Therefore, the gradual increase of *scpI* and *cppA* expression over the growth phases under iron-limited condition (chapter four) supports the reduction of phagocytosis in Aviles's experiment. Indeed, *S. iniae* does not possess the GAS-like Mga locus (Locke et al., 2008) containing *mga*, *emm*, and *scpA* in the regulon. Rather the *mga*-like *mgx* is located adjacently upstream of the *emm*-like *simA*, the *scpI* locates at the different locus in the genome and does not possess the upstream promoter region like *scpA* does for Mga in GAS (Locke et al., 2008). Therefore, the regulator of *scpI* and whether it is sensitive with iron limitation like *mga* is still unknown. Since the environmental cues and the growth phase related signals significantly influence bacterial

virulent gene expression, the expression of *scpI* and *cppA* is worth further investigation in different stress conditions.

In conclusion, I have demonstrated the influence of the growth phase on the expression of two complement proteases ScpI and CppA, and their coordinate regulation with CpsE, in the capsular operon in *S. iniae*. However which environmental and/or growth phase related signals that trigger this regulation are unknown. I also demonstrated that ScpI and CppA might be involved in interference with the classical complement pathway through the susceptibility of the knock out mutants to antiserum in the presence of complement. However, completely deleting these proteases revealed pleiotropic effects, which included bacterial growth as well as innate immune resistance, which suggests complicated multiple roles in pathogenesis and survival. These pleiotropic roles coupled with the understanding of the regulation of these proteins demonstrated in this thesis suggests that they are good candidates for conserved protein vaccine targets, and can be up-regulated during culture potentially improving potency and longevity of killed cell vaccines.

6. Chapter VI. References

Adamou, J.E., Wizemann, T.M., Barren, P., Langermann, S., 1998. Adherence of Streptococcus pneumoniae to Human Bronchial Epithelial Cells (BEAS-2B). Infection and immunity 66, 820-822.

Agnew, W., Barnes, A.C., 2007. Streptococcus iniae: an aquatic pathogen of global veterinary significance and a challenging candidate for reliable vaccination. Veterinary microbiology 122, 1-15.

Allard, M., Moisan, H., Brouillette, É., Gervais, A.L., Jacques, M., Lacasse, P., Diarra, M.S., Malouin, F., 2006. Transcriptional modulation of some Staphylococcus aureus iron-regulated genes during growth in vitro and in a tissue cage model in vivo. Microbes and Infection 8, 1679-1690.

Anderson, E.T., Wetherell, M.G., Winter, L.A., Olmsted, S.B., Cleary, P.P., Matsuka, Y.V., 2002. Processing, stability, and kinetic parameters of C5a peptidase from Streptococcus pyogenes. Eur J Biochem 269, 4839-4851.

Angel, C.S., Ruzek, M., Hostetter, M.K., 1994. Degradation of C3 by Streptococcus pneumoniae. J Infect Dis 170, 600-608.

Austin, B., Austin, D.A., 2012. Bacterial Fish Pathogens: Disease of Farmed and Wild Fish. Springer Netherlands.

Aviles, F., Zhang, M.M., Chan, J., Delamare-Deboutteville, J., Green, T.J., Dang, C., Barnes, A.C., 2013. The conserved surface M-protein SiMA of Streptococcus iniae is not effective as a cross-protective vaccine against differing capsular serotypes in farmed fish. Veterinary Microbiology 162, 151-159.

Bacun-Druzina, V., Butorac, A., Mrvcic, J., Dragicevic, T.L., Stehlik-Tomas, V., 2011. Bacterial stationary-phase evolution. Food Technology and Biotechnology 49, 13.

Baiano, J.C., Barnes, A.C., 2009. Towards control of Streptococcus iniae. Emerg Infect Dis 15, 1891-1896.

Baiano, J.C.F., Tumbol, R.A., Umapathy, A., Barnes, A.C., 2008. Identification and molecular characterisation of a fibrinogen binding protein from Streptococcus iniae. BMC Microbiology 8, 67-67.

Banerjee, A., Van Sorge, N.M., Sheen, T.R., Uchiyama, S., Mitchell, T.J., Doran, K.S., 2010. Activation of brain endothelium by pneumococcal neuraminidase NanA promotes bacterial internalization. Cell Microbiol 12, 1576-1588.

Barnes, A.C., Ellis, A.E., 2004. Role of capsule in serotypic differences and complement fixation by Lactococcus garvieae. Fish & Shellfish Immunology 16, 207-214.

Barnes, A.C., Young, F.M., Horne, M.T., Ellis, A.E., 2003. Streptococcus iniae: serological differences, presence of capsule and resistance to immune serum killing. Diseases of aquatic organisms 53, 241-247.

Barnett, T.C., Bugrysheva, J.V., Scott, J.R., 2007. Role of mRNA Stability in Growth Phase Regulation of Gene Expression in the Group A Streptococcus. Journal of Bacteriology 189, 1866-1873.

Barnum, S.R., Ames, R.S., Maycox, P.R., Hadingham, S.J., Meakin, J., Harrison, D., Parsons, A.A., 2002. Expression of the complement C3a and C5a receptors after permanent focal ischemia: An alternative interpretation. Glia 38, 169-173.

Becker, E.L., 1972. The relationship of the chemotactic behavior of the complement-derived factors, C3a, C5a, and C567, and a bacterial chemotactic factor to their ability to activate the proesterase 1 of rabbit polymorphonuclear leukocytes. The Journal of experimental medicine 135, 376-387.

Beckmann, C., Waggoner, J.D., Harris, T.O., Tamura, G.S., Rubens, C.E., 2002. Identification of novel adhesins from Group B streptococci by use of phage display reveals that C5a peptidase mediates fibronectin binding. Infect Immun 70, 2869-2876.

Behnsen, J., Lessing, F., Schindler, S., Wartenberg, D., Jacobsen, I.D., Thoen, M., Zipfel, P.F., Brakhage, A.A., 2010. Secreted Aspergillus fumigatus Protease Alp1 Degrades Human Complement Proteins C3, C4, and C5. Infection and immunity 78, 3585-3594.

Bell, C., 2000. The Microbiological Safety and Quality of Food Volumes I and II. International Food Safety News 9, 12.

Belotserkovsky, I., Dan-Goor, M., Hanski, E., Peled, A., Mishalian, I., Eran, Y., Hidalgo-Grass, C., Nizet, V., 2006. A streptococcal protease that degrades CXC chemokines and impairs bacterial clearance from infected tissues. The EMBO journal 25, 4628-4637.

Berry, A.M., Paton, J.C., 1996. Sequence heterogeneity of PsaA, a 37-kilodalton putative adhesin essential for virulence of Streptococcus pneumoniae. Infection and immunity 64, 5255-5262.

Beutler, B., 2004. Innate immunity: an overview. Molecular immunology 40, 845-859.

Birnboim, H.C., 1983. A rapid alkaline extraction method for the isolation of plasmid DNA. Methods in enzymology 100, 243-255.

Blom, A.M., Hallstrom, T., Riesbeck, K., 2009. Complement evasion strategies of pathogens-acquisition of inhibitors and beyond. Molecular immunology 46, 2808-2817.

Bohnsack, J.F., Chang, J.K., Hill, H.R., 1993. Restricted ability of group B streptococcal C5a-ase to inactivate C5a prepared from different animal species. Infection and immunity 61, 1421-1426.

Bonar, C.J., Wagner, R.A., 2003. A third report of "golf ball disease" in an Amazon River dolphin (Inia geoffrensis) associated with Streptococcus iniae. J Zoo Wildl Med 34, 296-301.

Boshra, H., Li, J., Sunyer, J.O., 2006. Recent advances on the complement system of teleost fish. Fish & shellfish immunology 20, 239-262.

Braga, V.M., 2002. Cell-cell adhesion and signalling. Curr Opin Cell Biol 14, 546-556.

Brightman, M.W., 1977. Morphology of blood-brain interfaces. Experimental Eye Research 25, 1-25.

Bromage, E.S., Owens, L., 2002. Infection of barramundi Lates calcarifer with Streptococcus iniae: effects of different routes of exposure. Diseases of aquatic organisms 52, 199-205.

Brown, C.K., Gu, Z.Y., Matsuka, Y.V., Purushothaman, S.S., Winter, L.A., Cleary, P.P., Olmsted, S.B., Ohlendorf, D.H., Earhart, C.A., 2005. Structure of the streptococcal cell wall C5a peptidase. Proc Natl Acad Sci U S A 102, 18391-18396.

Brown, E.J., Joiner, K.A., Gaither, T.A., Hammer, C.H., Frank, M.M., 1983. The interaction of C3b bound to pneumococci with factor H (beta 1H globulin), factor I (C3b/C4b inactivator), and properdin factor B of the human complement system. Journal of immunology (Baltimore, Md. : 1950) 131, 409-415.

Bsat, N., Herbig, A., Casillas-Martinez, L., Setlow, P., Helmann, J.D., 1998. Bacillus subtilis contains multiple Fur homologues: identification of the iron uptake (Fur) and peroxide regulon (PerR) repressors. Mol Microbiol 29, 189-198.

Buchanan, J.T., Colvin, K.M., Vicknair, M.R., Patel, S.K., Timmer, A.M., Nizet, V., 2008. Strain-associated virulence factors of Streptococcus iniae in hybrid-striped bass. Vet Microbiol 131, 145-153.

Buchanan, J.T., Stannard, J.A., Lauth, X., Ostland, V.E., Powell, H.C., Westerman, M.E., Nizet, V., 2005. Streptococcus iniae Phosphoglucomutase Is a Virulence Factor and a Target for Vaccine Development. Infection and Immunity 73, 6935-6944.

Buchmann, K., 1998. Binding and lethal effect of complement from Oncorhynchus mykiss on Gyrodactylus derjavini (Platyhelminthes: Monogenea). Diseases of aquatic organisms 32, 195-200.

Buchmann, K., Sigh, J., Nielsen, C.V., Dalgaard, M., 2001. Host responses against the fish parasitizing ciliate Ichthyophthirius multifiliis. Veterinary parasitology 100, 105-116.

Burns, J.E.H., Marciel, A.M., Musser, J.M., 1996. Activation of a 66-kilodalton human endothelial cell matrix metalloprotease by Streptococcus pyogenes extracellular cysteine protease. Infection and immunity 64, 4744-4750.

Byon, J.Y., Ohira, T., Hirono, I., Aoki, T., 2006. Comparative immune responses in Japanese flounder, Paralichthys olivaceus after vaccination with viral hemorrhagic septicemia virus (VHSV) recombinant glycoprotein and DNA vaccine using a microarray analysis. Vaccine 24, 921-930.

Caparon, M.G., Geist, R.T., Perez-Casal, J., Scott, J.R., 1992. Environmental regulation of virulence in group A streptococci: transcription of the gene encoding M protein is stimulated by carbon dioxide. Journal of Bacteriology 174, 5693-5701.

Caparon, M.G., Scott, J.R., 1987. Identification of a gene that regulates expression of M protein, the major virulence determinant of group A streptococci. Proceedings of the National Academy of Sciences of the United States of America 84, 8677-8681.

Carroll, M.C., 2000. The role of complement in B cell activation and tolerance. Advances in immunology 74, 61.

Cassat, J.E., Skaar, E.P., 2013. Iron in infection and immunity. Cell Host Microbe 13, 509-519.

Cecchelli, R., Berezowski, V., Lundquist, S., Culot, M., Renftel, M., Dehouck, M.P., Fenart, L., 2007. Modelling of the blood-brain barrier in drug discovery and development. Nature reviews. Drug discovery 6, 650-661.

Chan N. Dieu, A.C.B., 2015. Coordinate inverse regulation of putative complement-degrading proteases scpi and cppa with capsule in fish pathogenic streptococcus iniae. Veterinary Microbiology.

Chaussee, M.S., Phillips, E.R., Ferretti, J.J., 1997. Temporal production of streptococcal erythrogenic toxin B (streptococcal cysteine proteinase) in response to nutrient depletion. Infect Immun 65, 1956-1959.

Chen, C.C., Cleary, P.P., 1990. Complete nucleotide sequence of the streptococcal C5a peptidase gene of Streptococcus pyogenes. J Biol Chem 265, 3161-3167.

Chen, L., Keramati, L., Helmann, J.D., 1995. Coordinate regulation of Bacillus subtilis peroxide stress genes by hydrogen peroxide and metal ions. Proceedings of the National Academy of Sciences 92, 8190-8194.

Cheng, Q., Carlson, B., Pillai, S., Eby, R., Edwards, L., Olmsted, S.B., Cleary, P., 2001. Antibody against surface-bound C5a peptidase is opsonic and initiates macrophage killing of group B streptococci. Infect Immun 69, 2302-2308.

Cheng, Q., Stafslien, D., Purushothaman, S.S., Cleary, P., 2002. The group B streptococcal C5a peptidase is both a specific protease and an invasin. Infect Immun 70, 2408-2413.

Chiang-Ni, C., Wang, C.-H., Tsai, P.-J., Chuang, W.-J., Lin, Y.-S., Lin, M.-T., Liu, C.-C., Wu, J.-J., 2006. Streptococcal pyrogenic exotoxin B causes mitochondria damage to polymorphonuclear cells preventing phagocytosis of group A streptococcus. Medical microbiology and immunology 195, 55-63.

Chmouryguina, II, Suvorov, A.N., Carlson, B., Cleary, P., 1997. Structural and functional similarity of C5a-ase enzymes from group A and B streptococci. Adv Exp Med Biol 418, 757-759.

Chmouryguina, I., Suvorov, A., Ferrieri, P., Cleary, P.P., 1996. Conservation of the C5a peptidase genes in group A and B streptococci. Infect Immun 64, 2387-2390.

Cleary, P.P., Matsuka, Y.V., Huynh, T., Lam, H., Olmsted, S.B., 2004. Immunization with C5a peptidase from either group A or B streptococci enhances clearance of group A streptococci from intranasally infected mice. Vaccine 22, 4332-4341.

Cleary, P.P., Prahbu, U., Dale, J.B., Wexler, D.E., Handley, J., 1992. Streptococcal C5a peptidase is a highly specific endopeptidase. Infection and Immunity 60, 5219-5223.

Cohen, Z., Ehret, M., Maitre, M., Hamel, E., 1995. Ultrastructural analysis of tryptophan hydroxylase immunoreactive nerve terminals in the rat cerebral cortex and hippocampus: their associations with local blood vessels. Neuroscience 66, 555-569.

Collard, C.D., Agah, A., Stahl, G.L., 1998. Complement activation following reoxygenation of hypoxic human endothelial cells: role of intracellular reactive oxygen species, NF-kappaB and new protein synthesis. Immunopharmacology 39, 39-50.

Cundell, D.R., Gerard, N.P., Gerard, C., Idanpaan-Heikkila, I., Tuomanen, E.I., 1995. Streptococcus pneumoniae anchor to activated human cells by the receptor for platelet-activating factor. Nature 377, 435-438.

Cunningham, M.W., 2000. Pathogenesis of Group A Streptococcal Infections. Clinical Microbiology Reviews 13, 470-511.

Dahinden, C.A., Bischoff, S.C., Brunner, T., Krieger, M., Takafuji, S., Deweck, A.L., 1991. REGULATION OF MEDIATOR RELEASE BY HUMAN BASOPHILS - IMPORTANCE OF THE SEQUENCE AND TIME OF ADDITION IN THE COMBINED ACTION OF DIFFERENT AGONISTS. INTERNATIONAL ARCHIVES OF ALLERGY AND APPLIED IMMUNOLOGY 94, 161-164.

Das, A., Asatryan, L., Reddy, M.A., Wass, C.A., Stins, M.F., Joshi, S., Bonventre, J.V., Kim, K.S., 2001. Differential role of cytosolic phospholipase A2 in the invasion of brain microvascular endothelial cells by Escherichia coli and Listeria monocytogenes. J Infect Dis 184, 732-737.

de Vries, F.P., van Der Ende, A., van Putten, J.P., Dankert, J., 1996. Invasion of primary nasopharyngeal epithelial cells by Neisseria meningitidis is controlled by phase variation of multiple surface antigens. Infect Immun 64, 2998-3006.

Dintilhac, A., Alloing, G., Granadel, C., Claverys, J.P., 1997. Competence and virulence of Streptococcus pneumoniae: Adc and PsaA mutants exhibit a requirement for Zn and Mn resulting from inactivation of putative ABC metal permeases. Molecular microbiology 25, 727-739.

DiScipio, R.G., Daffern, P.J., Schraufstätter, I.U., Sriramarao, P., 1998. Human polymorphonuclear leukocytes adhere to complement factor H through an interaction that involves alphaMbeta2 (CD11b/CD18). Journal of immunology (Baltimore, Md.: 1950) 160, 4057.

Doran, K.S., Engelson, E.J., Khosravi, A., Maisey, H.C., Fedtke, I., Equils, O., Michelsen, K.S., Arditi, M., Peschel, A., Nizet, V., 2005. Blood-brain barrier invasion by group B Streptococcus depends upon proper cell-surface anchoring of lipoteichoic acid. J Clin Invest 115, 2499-2507.

Doran, K.S., Liu, G.Y., Nizet, V., 2003. Group B streptococcal beta-hemolysin/cytolysin activates neutrophil signaling pathways in brain endothelium and contributes to development of meningitis. J Clin Invest 112, 736-744.

Dukan, S., Nyström, T., 1998. Bacterial senescence: Stasis results in increased and differential oxidation of cytoplasmic proteins leading to developmental induction of the heat shock regulon. Genes and Development 12, 3431-3441.

Egesten, A., Olin, A.I., Linge, H.M., Yadav, M., Morgelin, M., Karlsson, A., Collin, M., 2009. SpeB of Streptococcus pyogenes differentially modulates antibacterial and receptor activating properties of human chemokines. PLoS One 4, e4769.

El-Naggar, A.K., Van Epps, D.E., Williams, J.R.C., 1980. Human-B and T-lymphocyte locomotion in response to casein, C5a, and f-met-leu-phe. Cellular immunology 56, 365-373.

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

Eldar, A., Bejerano, Y., Livoff, A., Horovitcz, A., Bercovier, H., 1995. Experimental streptococcal meningo-encephalitis in cultured fish. Veterinary Microbiology 43, 33-40.

Eldar, A., 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., Horovitcz, A., Bercovier, H., 1997. Development and efficacy of a vaccine against Streptococcus iniae infection in farmed rainbow trout. Veterinary immunology and immunopathology 56, 175-183.

Elliott, S.D., 1945. A PROTEOLYTIC ENZYME PRODUCED BY GROUP A STREPTOCOCCI WITH SPECIAL REFERENCE TO ITS EFFECT ON THE TYPE-SPECIFIC M ANTIGEN. The Journal of experimental medicine 81, 573-592.

Elsner, J., Oppermann, M., Czech, W., Dobos, G., Schöpf, E., Norgauer, J., Kapp, A., 1994. C3a activates reactive oxygen radical species production and intracellular calcium transients in human eosinophils. European journal of immunology 24, 518-522.

Evans, J.J., Shoemaker, C.A., Klesius, P.H., 2000. Experimental Streptococcus iniae infection of hybrid striped bass (Morone chrysops×Morone saxatilis) and tilapia (Oreochromis niloticus) by nares inoculation. Aquaculture 189, 197-210.

Eyngor, M., Lublin, A., Shapira, R., Hurvitz, A., Zlotkin, A., Tekoah, Y., Eldar, A., 2010. A pivotal role for the Streptococcus iniae extracellular polysaccharide in triggering proinflammatory cytokines transcription and inducing death in rainbow trout. FEMS Microbiol Lett 305, 109-120.

Facklam, R., Elliott, J., Shewmaker, L., Reingold, A., 2005. Identification and characterization of sporadic isolates of Streptococcus iniae isolated from humans. Journal of clinical microbiology 43, 933-937.

Faulkner, M.J., Helmann, J.D., 2011. Peroxide stress elicits adaptive changes in bacterial metal ion homeostasis. Antioxid Redox Signal 15, 175-189.

Ferretti, J.J., McShan, W.M., Ajdic, D., Savic, D.J., Savic, G., Lyon, K., Primeaux, C., Sezate, S., Suvorov, A.N., Kenton, S., Lai, H.S., Lin, S.P., Qian, Y., Jia, H.G., Najar, F.Z., Ren, Q., Zhu, H., Song, L., White, J., Yuan, X., Clifton, S.W., Roe, B.A., McLaughlin, R., 2001. Complete genome sequence of an M1 strain of Streptococcus pyogenes. Proc Natl Acad Sci U S A 98, 4658-4663.

Fischer, E., Sauer, U., 2005. Large-scale in vivo flux analysis shows rigidity and suboptimal performance of Bacillus subtilis metabolism. Nature genetics 37, 636-640.

Fischer Walker, C., Black, R.E., 2004. Zinc and the risk for infectious disease. Annual review of nutrition 24, 255-275.

Fischer, W.H., Hugli, T.E., 1997. Regulation of B cell functions by C3a and C3a(desArg): suppression of TNF-alpha, IL-6, and the polyclonal immune response. Journal of immunology (Baltimore, Md. : 1950) 159, 4279-4286.

Fischetti, V.A., 1989. Streptococcal M protein: molecular design and biological behavior. Clinical microbiology reviews 2, 285-314.

Fleming, S.D., Shea-Donohue, T., Guthridge, J.M., Kulik, L., Waldschmidt, T.J., Gipson, M.G., Tsokos, G.C., Holers, V.M., 2002. Mice deficient in complement receptors 1 and 2 lack a tissue injury-inducing subset of the natural antibody repertoire. Journal of immunology (Baltimore, Md. : 1950) 169, 2126-2133.

Framson, P.E., Nittayajarn, A., Merry, J., Youngman, P., Rubens, C.E., 1997. New genetic techniques for group B streptococci: high-efficiency transformation, maintenance of temperature-sensitive pWV01 plasmids, and mutagenesis with Tn917. Applied and Environmental Microbiology 63, 3539-3547.

Francis, K., Lewis, B.M., Akatsu, H., Monk, P.N., Cain, S.A., Scanlon, M.F., Morgan, B.P., Ham, J., Gasque, P., 2003. Complement C3a receptors in the pituitary gland: a novel pathway by which an innate immune molecule releases hormones involved in the control of inflammation. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 17, 2266-2266.

Fuangthong, M., Herbig, A.F., Bsat, N., Helmann, J.D., 2002. Regulation of the Bacillus subtilis fur and perR genes by PerR: not all members of the PerR regulon are peroxide inducible. J Bacteriol 184, 3276-3286.

Fuller, J.D., Bast, D.J., Nizet, V., Low, D.E., Joyce, C.S.d.A., 2001. Streptococcus iniae Virulence Is Associated with a Distinct Genetic Profile. Infection and immunity 69, 1994-2000.

Gasque, P., Singhrao, S.K., Neal, J.W., Wang, P., Sayah, S., Fontaine, M., Morgan, B.P., 1998. The receptor for complement anaphylatoxin C3a is expressed by myeloid cells and nonmyeloid cells in inflamed human central nervous system: analysis in multiple sclerosis and bacterial meningitis. Journal of immunology (Baltimore, Md. : 1950) 160, 3543-3554.

Ge, R., Sun, X., He, Q., 2009. Iron acquisition by Streptococcus species: An updated review. Frontiers of Biology in China 4, 392-401.

Gerard, N.P., Gerard, C., 2002. Complement in allergy and asthma. Current Opinion in Immunology 14, 705-708.

Geyer, A., Schmidt, K.H., 2000. Genetic organisation of the M protein region in human isolates of group C and G streptococci: two types of multigene regulator-like (mgrC) regions. Mol Gen Genet 262, 965-976.

Giannakis, E., Jokiranta, T.S., Male, D.A., Ranganathan, S., Ormsby, R.J., Fischetti, V.A., Mold, C., Gordon, D.L., 2003. A common site within factor H SCR 7 responsible for binding heparin, C-reactive protein and streptococcal M protein. European journal of immunology 33, 962-969.

Glauser, M.P., Zanetti, G., Baumgartner, J.D., Cohen, J., 1991. Septic shock: pathogenesis. The Lancet 338, 732-736.

Goldstein, I.M., Weissmann, G., 1974. Generation of C5-derived lysosomal enzyme-releasing activity (C5a) by lysates of leukocyte lysosomes. Journal of immunology (Baltimore, Md. : 1950) 113, 1583.

Gonzalez, S.F., Buchmann, K., Nielsen, M.E., 2007. Complement expression in common carp (Cyprinus carpio L.) during infection with Ichthyophthirius multifiliis. Developmental and comparative immunology 31, 576-586.

Gorski, J.P., Hugli, T.E., Müller-Eberhard, H.J., 1979. C4a: the third anaphylatoxin of the human complement system. Proceedings of the National Academy of Sciences of the United States of America 76, 5299-5302.

Green, J., Rolfe, M.D., Smith, L.J., 2014. Transcriptional regulation of bacterial virulence gene expression by molecular oxygen and nitric oxide. Virulence 5, 794-809.

Greiffenberg, L., Goebel, W., Kim, K.S., Weiglein, I., Bubert, A., Engelbrecht, F., Stins, M., Kuhn, M., 1998. Interaction of Listeria monocytogenes with human brain microvascular endothelial cells: InlB-dependent invasion, long-term intracellular growth, and spread from macrophages to endothelial cells. Infect Immun 66, 5260-5267.

Griffiths, B.B., McClain, O., 1988. The role of iron in the growth and hemolysin (Streptolysin S) production in Streptococcus pyogenes. Journal of basic microbiology 28, 427-436.

Guedon, E., Delorme, C., Pons, N., Cruaud, C., Loux, V., Couloux, A., Gautier, C., Sanchez, N., Layec, S., Galleron, N., Almeida, M., van de Guchte, M., Kennedy, S.P., Ehrlich, S.D., Gibrat, J.F., Wincker, P., Renault, P., 2011. Complete genome sequence of the commensal Streptococcus salivarius strain JIM8777. J Bacteriol 193, 5024-5025.

Guo, R.-F., Ward, P.A., 2005. Role of C5a in inflammatory responses. Annual review of immunology 23, 821-852.

Gustafsson, M.C.U., Lannergård, J., Nilsson, O.R., Kristensen, B.M., Olsen, J.E., Harris, C.L., Ufret-Vincenty, R.L., Stålhammar-Carlemalm, M., Lindahl, G., Department of Laboratory Medicine, L., Sektionen för medicinsk, m., Faculty of, M., Division of Medical, M., Lunds, u., Institutionen för laboratoriemedicin, L., Medicin, Lund, U., 2013. Factor H binds to the hypervariable region of many Streptococcus pyogenes M proteins but does not promote phagocytosis resistance or acute virulence. PLoS pathogens 9, e1003323.

Haas, P.J., van Strijp, J., 2007. Anaphylatoxins: their role in bacterial infection and inflammation. Immunol Res 37, 161-175.

Hammerschmidt, S., Muller, A., Sillmann, H., Muhlenhoff, M., Borrow, R., Fox, A., van Putten, J., Zollinger, W.D., Gerardy-Schahn, R., Frosch, M., 1996. Capsule phase variation in Neisseria meningitidis serogroup B by slipped-strand mispairing in the polysialyltransferase gene (siaD): correlation with bacterial invasion and the outbreak of meningococcal disease. Mol Microbiol 20, 1211-1220.

Ha□nsch, G.M., Till, G.O., Rother, K.O., 1998. The complement system. Springer, New York.

Harris, P.D., Soleng, A., Bakke, T.A., 1998. Killing of Gyrodactylus salaris (Platyhelminthes, Monogenea) mediated by host complement. Parasitology 117 (Pt 2), 137-143.

Hartmann, K., Henz, B.M., Krüger-Krasagakes, S., Köhl, J., Burger, R., Guhl, S., Haase, I., Lippert, U., Zuberbier, T., 1997. C3a and C5a stimulate chemotaxis of human mast cells. Blood 89, 2863-2870.

Haviland, D.L., Wetsel, R.A., McCoy, R.L., Whitehead, W.T., Akama, H., Molmenti, E.P., Brown, A., Haviland, J.C., Parks, W.C., Perlmutter, D.H., 1995. Cellular expression of the C5a anaphylatoxin receptor (C5aR): demonstration of C5aR on nonmyeloid cells of the liver and lung. The Journal of Immunology 154, 1861-1869.

Hawlisch, H., Wills-Karp, M., Karp, C.L., Köhl, J., 2004. The anaphylatoxins bridge innate and adaptive immune responses in allergic asthma. Molecular immunology 41, 123-131.

Hellemans, J., Mortier, G., De Paepe, A., Speleman, F., Vandesompele, J., 2007. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. Genome Biol 8, R19.

Herwald, H., Collin, M., Müller-Esterl, W., Björck, L., Lund, U., Avdelningen för, i., Department of Clinical Sciences, L., Sektion, III, Division of Infection, M., Institutionen för kliniska vetenskaper, L., Lunds, u., Division, III, Faculty of, M., Medicin, 1996. Streptococcal cysteine proteinase releases kinins: a virulence mechanism. The Journal of experimental medicine 184, 665-673.

Higgins, C.F., 2001. ABC transporters: physiology, structure and mechanism – an overview. Research in Microbiology 152, 205-210.

Hill, H.R., Bohnsack, J.F., Morris, E.Z., Augustine, N.H., Parker, C.J., Cleary, P.P., Wu, J.T., 1988. Group B streptococci inhibit the chemotactic activity of the fifth component of complement. Journal of immunology (Baltimore, Md. : 1950) 141, 3551-3556.

Hoch, J.A., 2000. Two-component and phosphorelay signal transduction. Current Opinion in Microbiology 3, 165-170.

Holden, M.T., Scott, A., Cherevach, I., Chillingworth, T., Churcher, C., Cronin, A., Dowd, L., Feltwell, T., Hamlin, N., Holroyd, S., Jagels, K., Moule, S., Mungall, K., Quail, M.A., Price, C., Rabbinowitsch, E., Sharp, S., Skelton, J., Whitehead, S., Barrell, B.G., Kehoe, M., Parkhill, J., 2007. Complete genome of acute rheumatic fever-associated serotype M5 Streptococcus pyogenes strain manfredo. J Bacteriol 189, 1473-1477.

Holland, M.C.H., Lambris, J.D., 2002. The complement system in teleosts. Fish & shellfish immunology 12, 399-420.

Hook, W.A., Siraganian, R.P., Wahl, S.M., 1975. Complement-induced histamine release from human basophils. I. Generation of activity in human serum. Journal of immunology (Baltimore, Md. : 1950) 114, 1185.

Hornef, M.W., Wick, M.J., Rhen, M., Normark, S., 2002. Bacterial strategies for overcoming host innate and adaptive immune responses. Nature immunology 3, 1033-1040.

Hostetter, M.K., Dunny, G., Nandiwada, L.S. 2004. Human complement C3-degrading protein from Streptococcus pneumoniae (Google Patents).

Huber-Lang, M., Sarma, J.V., Zetoune, F.S., Rittirsch, D., Neff, T.A., McGuire, S.R., Lambris, J.D., Warner, R.L., Flierl, M.A., Hoesel, L.M., Gebhard, F., Younger, J.G., Drouin, S.M., Wetsel, R.A., Ward, P.A., 2006. Generation of C5a in the absence of C3: a new complement activation pathway. Nat Med 12, 682-687.

Hull, J.R., Tamura, G.S., Castner, D.G., 2008. Interactions of the streptococcal C5a peptidase with human fibronectin. Acta biomaterialia 4, 504-513.

Hung, C.H., Tsao, N., Zeng, Y.F., Lu, S.L., Chuan, C.N., Lin, Y.S., Wu, J.J., Kuo, C.F., 2012. Synergistic effects of streptolysin S and streptococcal pyrogenic exotoxin B on the mouse model of group A streptococcal infection. Med Microbiol Immunol 201, 357-369.

Husmann, L.K., Yung, D.L., Hollingshead, S.K., Scott, J.R., 1997. Role of putative virulence factors of Streptococcus pyogenes in mouse models of long-term throat colonization and pneumonia. Infection and immunity 65, 1422-1430.

Hyams, C., Trzcinski, K., Camberlein, E., Weinberger, D.M., Chimalapati, S., Noursadeghi, M., Lipsitch, M., Brown, J.S., 2013. Streptococcus pneumoniae Capsular Serotype Invasiveness Correlates with the Degree of Factor H Binding and Opsonization with C3b/iC3b. Infection and immunity 81, 354-363.

Imlay, J.A., 2003. Pathways of oxidative damage. Annu Rev Microbiol 57, 395-418.

Imlay, J.A., 2008. Cellular defenses against superoxide and hydrogen peroxide. Annu Rev Biochem 77, 755-776.

Jain, S.K., Paul-Satyaseela, M., Lamichhane, G., Kim, K.S., Bishai, W.R., 2006. Mycobacterium tuberculosis invasion and traversal across an in vitro human blood-brain barrier as a pathogenic mechanism for central nervous system tuberculosis. J Infect Dis 193, 1287-1295.

Jakubovics, N.S., Jenkinson, H.F., 2001. Out of the iron age: new insights into the critical role of manganese homeostasis in bacteria. Microbiology (Reading, England) 147, 1709-1718.

Jakubovics, N.S., Smith, A.W., Jenkinson, H.F., 2000. Expression of the virulence-related Sca (Mn2+) permease in Streptococcus gordonii is regulated by a diphtheria toxin metallorepressor-like protein ScaR. Mol Microbiol 38, 140-153.

Janeway, C.A., 2001. Immunobiology: the immune system in health and disease. Garland, New York.

Janulczyk, R., Pallon, J., Björck, L., 1999. Identification and characterization of a Streptococcus pyogenes ABC transporter with multiple specificity for metal cations. Molecular Microbiology 34, 596-606.

Janulczyk, R., Rasmussen, M., 2001. Improved pattern for genome-based screening identifies novel cell wall-attached proteins in gram-positive bacteria. Infect Immun 69, 4019-4026.

Jenkins, J.A., Ourth, D.D., 1993. Opsonic effect of the alternative complement pathway on channel catfish peripheral blood phagocytes. Veterinary Immunology and Immunopathology 39, 447-459.

Jensen, L.B., Koch, C., 1991. Genetic polymorphism of component C3 of rainbow trout (Oncorhynchus mykiss) complement. Fish and Shellfish Immunology 1, 237-242.

Ji, Y., McLandsborough, L., Kondagunta, A., Cleary, P.P., 1996. C5a peptidase alters clearance and trafficking of group A streptococci by infected mice. Infection and immunity 64, 503-510.

Johanson, C.E., 1980. Permeability and vascularity of the developing brain: cerebellum vs cerebral cortex. Brain research 190, 3-16.

Jones, K.F., Fischetti, V.A., 1988. The importance of the location of antibody binding on the M6 protein for opsonization and phagocytosis of group A M6 streptococci. The Journal of experimental medicine 167, 1114-1123.

Kadioglu, A., Taylor, S., Iannelli, F., Pozzi, G., Mitchell, T.J., Andrew, P.W., 2002. Upper and Lower Respiratory Tract Infection by Streptococcus pneumoniae Is Affected by Pneumolysin Deficiency and Differences in Capsule Type. Infection and immunity 70, 2886-2890.

Kaige, N., Mjyazaki, T., Kubota, S.S., 1984. The Pathogen and the Histopathology of Vertebral Deformity in Cultured Yellowtail. Fish Pathology 19, 173-179.

Kapur, V., Majesky, M.W., Li, L.L., Black, R.A., Musser, J.M., 1993a. Cleavage of interleukin 1 beta (IL-1 beta) precursor to produce active IL-1 beta by a conserved extracellular cysteine protease from Streptococcus pyogenes. Proceedings of the National Academy of Sciences of the United States of America 90, 7676-7680.

Kapur, V., Topouzis, S., Majesky, M.W., Li, L.L., Hamrick, M.R., Hamill, R.J., Patti, J.M., Musser, J.M., 1993b. A conserved Streptococcus pyogenes extracellular cysteine protease cleaves human fibronectin and degrades vitronectin. Microb Pathog 15, 327-346.

Kay, A.B., Shin, H.S., Austen, K.F. 1973. Selective attraction of eosinophils and synergism between eosinophil chemotactic factor of anaphylaxis (ECF-A) and a fragment cleaved from the fifth component of complement (C5a). In Immunology (ENGLAND), 969-976.

Kaya, Z., Afanasyeva, M., Wang, Y., Dohmen, K.M., Schlichting, J., Tretter, T., Fairweather, D., Holers, V.M., Rose, N.R., 2001. Contribution of the innate immune system to autoimmune myocarditis: a role for complement. Nature immunology 2, 739-745.

Kelley, L.A., Sternberg, M.J., 2009. Protein structure prediction on the Web: a case study using the Phyre server. Nature protocols 4, 363-371.

Kim, K.S., Itabashi, H., Gemski, P., Sadoff, J., Warren, R.L., Cross, A.S., 1992. The K1 capsule is the critical determinant in the development of Escherichia coli meningitis in the rat. The Journal of clinical investigation 90, 897-905.

Klumpp, S., Zhang, Z., Hwa, T., 2009. Growth Rate-Dependent Global Effects on Gene Expression in Bacteria. Cell 139, 1366-1375.

Kreikemeyer, B., McIver, K.S., Podbielski, A., 2003. Virulence factor regulation and regulatory networks in Streptococcus pyogenes and their impact on pathogen–host interactions. Trends in Microbiology 11, 224-232.

Kuo, C.-F., Lin, Y.-S., Chuang, W.-J., Wu, J.-J., Tsao, N., 2008. Degradation of Complement 3 by Streptococcal Pyrogenic Exotoxin B Inhibits Complement Activation and Neutrophil Opsonophagocytosis. Infection and Immunity 76, 1163-1169.

Kvennefors, E.C., Leggat, W., Kerr, C.C., Ainsworth, T.D., Hoegh-Guldberg, O., Barnes, A.C., 2010. Analysis of evolutionarily conserved innate immune components in coral links immunity and symbiosis. Dev Comp Immunol 34, 1219-1229.

Lachmann, P.J., Thompson, R.A., 1970. Reactive lysis: the complement-mediated lysis of unsensitized cells. II. The characterization of activated reactor as C56 and the participation of C8 and C9. The Journal of experimental medicine 131, 643-657.

Lammens, M., Decostere, A., Haesebrouck, F., 2000. Effect of Flavobacterium psychrophilum strains and their metabolites on the oxidative activity of rainbow trout oncorhynchus mykiss phagocytes. Diseases of aquatic organisms 41, 173-179.

Lars, B., Francesco, I., Robert, J., Gianni, P., Anders, S. 2002. Fh-binding protein of streptococcus pneumoniae (Google Patents).

Laskin, D.L., Pilaro, A.M., 1986. Potential role of activated macrophages in acetaminophen hepatotoxicity. I. Isolation and characterization of activated macrophages from rat liver. Toxicology and applied pharmacology 86, 204-215.

Lau, S.K.P., Woo, P.C.Y., Tse, H., Leung, K.-W., Wong, S.S.Y., Yuen, K.-Y., 2003. Invasive Streptococcus iniae infections outside North America. Journal of clinical microbiology 41, 1004-1009.

LeBlanc, D.J., Inamine, J.M., Lee, L.N., 1986. Broad geographical distribution of homologous erythromycin, kanamycin, and streptomycin resistance determinants among group D streptococci of human and animal origin. Antimicrobial agents and chemotherapy 29, 549-555.

LeBlanc, D.J., Lee, L.N., Inamine, J.M., 1991. Cloning and nucleotide base sequence analysis of a spectinomycin adenyltransferase AAD(9) determinant from Enterococcus faecalis. Antimicrobial Agents and Chemotherapy 35, 1804-1810.

Leib, S.L., Täuber, M.G., 1999. PATHOGENESIS OF BACTERIAL MENINGITIS. Infectious disease clinics of North America 13, 527-548.

Lin, A., Loughman, J.A., Zinselmeyer, B.H., Miller, M.J., Caparon, M.G., 2009. Streptolysin S inhibits neutrophil recruitment during the early stages of Streptococcus pyogenes infection. Infect Immun 77, 5190-5201.

Liszewski, M.K., Farries, T.C., Lublin, D.M., Rooney, I.A., Atkinson, J.P., 1996. Control of the complement system. Adv Immunol 61, 201-283.

Litwin, C.M., Calderwood, S.B., 1993. Role of iron in regulation of virulence genes. Clinical Microbiology Reviews 6, 137-149.

Locke, J.B., Aziz, R.K., Vicknair, M.R., Nizet, V., Buchanan, J.T., 2008. <italic>Streptococcus iniae</italic> 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., Buchanan, J.T., 2007. Streptococcus iniae Capsule Impairs Phagocytic Clearance and Contributes to Virulence in Fish. The Journal of Bacteriology 189, 1279-1287.

Loughman, J.A., Caparon, M., 2006. Regulation of SpeB in Streptococcus pyogenes by pH and NaCl: a model for in vivo gene expression. J Bacteriol 188, 399-408.

Lowe, B.A., Miller, J.D., Neely, M.N., 2007. Analysis of the polysaccharide capsule of the systemic pathogen Streptococcus iniae and its implications in virulence. Infect Immun 75, 1255-1264.

Lukomski, S., Sreevatsan, S., Amberg, C., Reichardt, W., Woischnik, M., Podbielski, A., Musser, J.M., 1997. Inactivation of Streptococcus pyogenes extracellular cysteine protease significantly decreases mouse lethality of serotype M3 and M49 strains. The Journal of clinical investigation 99, 2574-2580.

Madigan, M.T., 2015. Brock biology of microorganisms. Pearson, Boston.

Magee, A.D., Yother, J., 2001. Requirement for capsule in colonization by Streptococcus pneumoniae. Infection and immunity 69, 3755-3761.

Mairey, E., Genovesio, A., Donnadieu, E., Bernard, C., Jaubert, F., Pinard, E., Seylaz, J., Olivo-Marin, J.C., Nassif, X., Dumenil, G., 2006. Cerebral microcirculation shear stress levels determine Neisseria meningitidis attachment sites along the blood-brain barrier. The Journal of experimental medicine 203, 1939-1950.

Mak, T.W., Saunders, M.E., 2007. THE IMMUNE RESPONSE: BASIC AND CLINICAL PRINCIPLES. Shock 27, 220-221.

Marchler-Bauer, A., Derbyshire, M.K., Gonzales, N.R., Lu, S., Chitsaz, F., Geer, L.Y., Geer, R.C., He, J., Gwadz, M., Hurwitz, D.I., Lanczycki, C.J., Lu, F., Marchler, G.H., Song, J.S., Thanki, N., Wang, Z., Yamashita, R.A., Zhang, D., Zheng, C., Bryant, S.H., 2015. CDD: NCBI's conserved domain database. Nucleic Acids Res 43, D222-226.

Marder, S.R., Chenoweth, D.E., Goldstein, I.M., Perez, H.D., 1985. Chemotactic responses of human peripheral blood monocytes to the complement-derived peptides C5a and C5a des Arg. The Journal of Immunology 134, 3325.

Maret, W., 2001. Zinc biochemistry, physiology, and homeostasis – recent insights and current trends. Biometals : an international journal on the role of metal ions in biology, biochemistry, and medicine 14, 187-190.

Martins, E.R., Melo-Cristino, J., Ramirez, M., 2010. Evidence for rare capsular switching in Streptococcus agalactiae. J Bacteriol 192, 1361-1369.

Matsuka, Y.V., Pillai, S., Gubba, S., Musser, J.M., Olmsted, S.B., 1999. Fibrinogen cleavage by the Streptococcus pyogenes extracellular cysteine protease and generation of antibodies that inhibit enzyme proteolytic activity. Infection and immunity 67, 4326-4333.

Matsushita, M., Endo, Y., Fujita, T., 2000. Cutting edge: complement-activating complex of ficolin and mannose-binding lectin-associated serine protease. Journal of immunology (Baltimore, Md. : 1950) 164, 2281-2284.

Matsuyama, H., Yano, T., Yamakawa, T., Nakao, M., 1992. Opsonic effect of the third complement component (C3) of carp (Cyprinus carpio) on phagocytosis by neutrophils. Fish and Shellfish Immunology 2, 69-78.

Matter, K., Balda, M.S., 2003. Signalling to and from tight junctions. Nat Rev Mol Cell Biol 4, 225-236.

Mayilyan, K.R., Kang, Y.H., Dodds, A.W., Sim, R.B. 2008. The Complement System in Innate Immunity, In: Springer Berlin Heidelberg, Berlin, Heidelberg, 219-236.

McDevitt, C.A., Ogunniyi, A.D., Valkov, E., Lawrence, M.C., Kobe, B., McEwan, A.G., Paton, J.C., 2011. A Molecular Mechanism for Bacterial Susceptibility to Zinc. PLoS Pathogens 7, e1002357.

McIver, K.S., Heath, A.S., Scott, J.R., 1995. Regulation of virulence by environmental signals in group A streptococci: influence of osmolarity, temperature, gas exchange, and iron limitation on emm transcription. Infection and immunity 63, 4540-4542.

McIver, K.S., Scott, J.R., 1997. Role of mga in growth phase regulation of virulence genes of the group A streptococcus. Journal of bacteriology 179, 5178-5187.

Michal, P., Jan, P., 2012. Protease-dependent mechanisms of complement evasion by bacterial pathogens. Biological Chemistry 393, 873-888.

Millard, C.M., Baiano, J.C., Chan, C., Yuen, B., Aviles, F., Landos, M., Chong, R.S., Benedict, S., Barnes, A.C., 2012. Evolution of the capsular operon of Streptococcus iniae in response to vaccination. Appl Environ Microbiol 78, 8219-8226.

Miller, J.D., Neely, M.N., 2005. Large-scale screen highlights the importance of capsule for virulence in the zoonotic pathogen Streptococcus iniae. Infect Immun 73, 921-934.

Molina, H., Holers, V.M., Li, B., Fang, Y.-F., Mariathasan, S., Goellner, J., Strauss-Schoenberger, J., Karr, R.W., Chaplin, D.D., 1996. Markedly Impaired Humoral Immune Response in Mice Deficient in Complement Receptors 1 and 2. Proceedings of the National Academy of Sciences of the United States of America 93, 3357-3361.

Monk, P.N., Scola, A.M., Madala, P., Fairlie, D.P., 2007. Function, structure and therapeutic potential of complement C5a receptors. British Journal of Pharmacology 152, 429-448.

Monsinjon, T., Gasque, P., Ischenko, A., Fontaine, M., 2001. C3A binds to the seven transmembrane anaphylatoxin receptor expressed by epithelial cells and triggers the production of IL-8. FEBS letters 487, 339-346.

Moritomo, T., Iida, T., Wakabayashi, H., 1988. Chemiluminescence of Neutrophils Isolated from Peripheral Blood of Eel. Fish Pathology 23, 49-53.

Morona, J.K., Morona, R., Paton, J.C., 2006. Attachment of capsular polysaccharide to the cell wall of Streptococcus pneumoniae type 2 is required for invasive disease. Proceedings of the National Academy of Sciences 103, 8505-8510.

Morona, R., Morona, J.K., Miller, D.C., Paton, J.C., 2004. The Effect That Mutations in the Conserved Capsular Polysaccharide Biosynthesis Genes cpsA, cpsB, and cpsD Have on Virulence of Streptococcus pneumoniae. The Journal of infectious diseases 189, 1905-1913.

Mouslim, C., Hughes, K.T., 2014. The effect of cell growth phase on the regulatory cross-talk between flagellar and Spi1 virulence gene expression. PLoS pathogens 10, e1003987.

Mühldorfer, I., Schäfer, K.P., 2001. Emerging bacterial pathogens, Vol 8. Karger, New York; Basel.

Müller-Eberhard, H.J., Schreiber, R.D. 1980. Molecular Biology and Chemistry of the Alternative Pathway of Complement1–3, In: Henry, G.K., Frank, J.D. (Eds.) Advances in Immunology. Academic Press, 1-53.

Mytilinaios, I., Salih, M., Schofield, H.K., Lambert, R.J.W., 2012. Growth curve prediction from optical density data. International Journal of Food Microbiology 154, 169-176.

Neidhardt, F.C., Ingraham, J.L., Schaechter, M., 1990. Physiology of the bacterial cell: a molecular approach. Sinauer Associates, Sunderland, Mass.

Neilands, J.B., 1981. Microbial iron compounds. Annual Review of Biochemistry 50, 715-731.

Neilands, J.B., 1982. Microbial envelope proteins related to iron. Annual Review of Microbiology 36, 285-309.

Nielsen, H., Brunak, S., von Heijne, G., 1999a. Machine learning approaches for the prediction of signal peptides and other protein sorting signals. Protein Engineering 12, 3-9.

Nielsen, H., Brunak, S., von Heijne, G., 1999b. Machine learning approaches for the prediction of signal peptides and other protein sorting signals. Protein engineering 12, 3.

Nikulin, J., Panzner, U., Frosch, M., Schubert-Unkmeir, A., 2006. Intracellular survival and replication of Neisseria meningitidis in human brain microvascular endothelial cells. Int J Med Microbiol 296, 553-558.

Nizet, V., Kim, K.S., Stins, M., Jonas, M., Chi, E.Y., Nguyen, D., Rubens, C.E., 1997. Invasion of brain microvascular endothelial cells by group B streptococci. Infection and Immunity 65, 5074-5081.

Nobbs, A.H., Lamont, R.J., Jenkinson, H.F., 2009. Streptococcus adherence and colonization. Microbiol Mol Biol Rev 73, 407-450, Table of Contents.

Nonaka, M., Irie, M., Tanabe, K., Kaidoh, T., Natsuume-Sakai, S., Takahashi, M., 1985. Identification and characterization of a variant of the third component of complement (C3) in rainbow trout (Salmo gairdneri) serum. Journal of Biological Chemistry 260, 809.

Norgauer, J., Dobos, G., Kownatzki, E., Dahinden, C., Burger, R., Kupper, R., Gierschik, P., 1993. Complement fragment C3a stimulates Ca2+ influx in neutrophils via a pertussis-toxin-sensitive G protein. European journal of biochemistry / FEBS 217, 289-294.

O'Connor, S.P., Cleary, P.P., 1987. In vivo Streptococcus pyogenes C5a Peptidase Activity: Analysis Using Transposon- and Nitrosoguanidine-Induced Mutants. The Journal of infectious diseases 156, 495-504.

Okada, N., Liszewski, M.K., Atkinson, J.P., Caparon, M., 1995. Membrane cofactor protein (CD46) is a keratinocyte receptor for the M protein of the group A streptococcus. Proc Natl Acad Sci U S A 92, 2489-2493.

Okumura, K., Shimomura, Y., Murayama, S.Y., Yagi, J., Ubukata, K., Kirikae, T., Miyoshi-Akiyama, T., 2012. Evolutionary paths of streptococcal and staphylococcal superantigens. BMC Genomics 13, 404.

Overturf, K., LaPatra, S., 2006. Quantitative expression (Walbaum) of immunological factors in rainbow trout, Oncorhynchus mykiss (Walbaum), after infection with either Flavobacterium psychrophilum, Aeromonas salmonicida, or infectious haematopoietic necrosis virus. J Fish Dis 29, 215-224.

Papadimitriou, K., Anastasiou, R., Mavrogonatou, E., Blom, J., Papandreou, N., Hamodrakas, S., Ferreira, S., Renault, P., Supply, P., Pot, B., Tsakalidou, E., 2014a. Comparative genomics of the dairy isolate Streptococcus macedonicus ACA-DC 198 against related members of the Streptococcus bovis/Streptococcus equinus complex. BMC Genomics 15, 272.

Papadimitriou, K., Anastasiou, R., Mavrogonatou, E., Blom, J., Papandreou, N.C., Hamodrakas, S.J., Ferreira, S., Renault, P., Supply, P., Pot, B., Tsakalidou, E., 2014b. Comparative genomics of the dairy isolate Streptococcus macedonicus ACA-DC 198 against related members of the Streptococcus bovis/Streptococcus equinus complex. BMC Genomics 15, 272.

Pardridge, W.M. 2003. Molecular Biology of the Blood-Brain Barrier, In, 385-399.

Paspalas, C.D., Papadopoulos, G.C., 1996. Ultrastructural relationships between noradrenergic nerve fibers and non-neuronal elements in the rat cerebral cortex. Glia 17, 133-146.

Paulson, O.B., Newman, E.A., 1987. Does the release of potassium from astrocyte endfeet regulate cerebral blood flow? Science 237, 896-898.

Pausa, M., Pellis, V., Cinco, M., Giulianini, P.G., Presani, G., Perticarari, S., Murgia, R., Tedesco, F., 2003. Serum-Resistant Strains of Borrelia burgdorferi Evade Complement-Mediated Killing by Expressing a CD59-Like Complement Inhibitory Molecule. The Journal of Immunology 170, 3214.

Pfaffl, M.W., Horgan, G.W., Dempfle, L., 2002. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic acids research 30, e36-36.

Pier, G.B., Madin, S.H., 1976. Streptococcus iniae sp. nov., a Beta-Hemolytic Streptococcus Isolated from an Amazon Freshwater Dolphin, Inia geoffrensis. International Journal of Systematic Bacteriology 26, 545-553.

Pier, G.B., Madin, S.H., Al-Nakeeb, S., 1978. Isolation and Characterization of a Second Isolate of Streptococcus iniae. International Journal of Systematic Bacteriology 28, 311-314.

Podack, E.R., Tschopp, J., 1982. Polymerization of the ninth component of complement (C9): formation of poly(C9) with a tubular ultrastructure resembling the membrane attack complex of complement. Proc Natl Acad Sci U S A 79, 574-578.

Prasadarao, N.V., Wass, C.A., Stins, M.F., Shimada, H., Kim, K.S., 1999. Outer membrane protein A-promoted actin condensation of brain microvascular endothelial cells is required for Escherichia coli invasion. Infect Immun 67, 5775-5783.

Pridgeon, J.W., Zhang, D., Zhang, L., 2014a. Complete Genome Sequence of a Virulent Strain, Streptococcus iniae ISET0901, Isolated from Diseased Tilapia. Genome Announc 2.

Pridgeon, J.W., Zhang, D., Zhang, L., 2014b. Complete Genome Sequence of the Attenuated Novobiocin-Resistant Streptococcus iniae Vaccine Strain ISNO. Genome Announc 2.

Prodeus, A.P., Goerg, S., Shen, L.M., Pozdnyakova, O.O., Chu, L., Alicot, E.M., Goodnow, C.C., Carroll, M.C., 1998. A critical role for complement in maintenance of self-tolerance. Immunity 9, 721-731.

Ram, S., McQuillen, D.P., Gulati, S., Elkins, C., Pangburn, M.K., Rice, P.A., 1998. Binding of Complement Factor H to Loop 5 of Porin Protein 1A: A Molecular Mechanism of Serum Resistance of Nonsialylated Neisseria gonorrhoeae. The Journal of experimental medicine 188, 671-680.

Ramu, P., Tanskanen, R., Holmberg, M., Lähteenmäki, K., Korhonen, T.K., Meri, S., 2007. The surface protease PgtE of Salmonella enterica affects complement activity by proteolytically cleaving C3b, C4b and C5. FEBS letters 581, 1716-1720.

Ransohoff, R.M., Kivisakk, P., Kidd, G., 2003. Three or more routes for leukocyte migration into the central nervous system. Nat Rev Immunol 3, 569-581.

Ratledge, C., Dover, L.G., 2000. IRON METABOLISM IN PATHOGENIC BACTERIA. Annual Reviews in Microbiology 54, 881-941.

Rato, M.G., Nerlich, A., Bergmann, R., Bexiga, R., Nunes, S.F., Vilela, C.L., Santos-Sanches, I., Chhatwal, G.S., 2011. Virulence gene pool detected in bovine group C Streptococcus dysgalactiae subsp. dysgalactiae isolates by use of a group A S. pyogenes virulence microarray. J Clin Microbiol 49, 2470-2479.

Reid, K.B., Turner, M.W., 1994. Mammalian lectins in activation and clearance mechanisms involving the complement system. Springer Semin Immunopathol 15, 307-326.

Ring, A., Weiser, J.N., Tuomanen, E.I., 1998. Pneumococcal trafficking across the blood-brain barrier. Molecular analysis of a novel bidirectional pathway. J Clin Invest 102, 347-360.

Rink, L., Gabriel, P., 2001. Extracellular and immunological actions of zinc. Biometals : an international journal on the role of metal ions in biology, biochemistry, and medicine 14, 367-383.

Rooijakkers, S.H.M., van Strijp, J.A.G., 2007. Bacterial complement evasion. Molecular immunology 44, 23-32.

Rosinski-Chupin, I., Sauvage, E., Mairey, B., Mangenot, S., Ma, L., Da Cunha, V., Rusniok, C., Bouchier, C., Barbe, V., Glaser, P., 2013. Reductive evolution in Streptococcus agalactiae and the emergence of a host adapted lineage. BMC Genomics 14, 252.

Sacks, T., Moldow, C.F., Craddock, P.R., Bowers, T.K., Jacob, H.S., 1978. Oxygen radicals mediate endothelial cell damage by complement-stimulated granulocytes. An in vitro model of immune vascular damage. The Journal of clinical investigation 61, 1161-1167.

Saeij, J.P.J., Bergsma, J., Nakayasu, C., Taverne-Thiele, J.J., Huttenhuis, B.T., Rombout, J.H.W.M., Grou, C.P.O., 2006. Ontogeny of the common carp (Cyprinus carpio L.) innate immune system. Developmental and Comparative Immunology 30, 557-574.

Sakai, D.K., 1984. Opsonization by fish antibody and complement in the immune phagocytosis by peritoneal exudate cells isolated from salmonid fishes. Journal of Fish Diseases 7, 29-38.

Schaechter, M., Maaloe, O., Kjeldgaard, N.O., 1958. Dependency on medium and temperature of cell size and chemical composition during balanced grown of Salmonella typhimurium. Journal of general microbiology 19, 592.

Schneewind, O., Fowler, A., Faull, K.F., 1995. Structure of the cell wall anchor of surface proteins in Staphylococcus aureus. Science 268, 103-106.

Schulze, C., Firth, J.A., 1993. Immunohistochemical localization of adherens junction components in blood-brain barrier microvessels of the rat. J Cell Sci 104 (Pt 3), 773-782.

Scott, M., Hwa, T., 2011. Bacterial growth laws and their applications. Current opinion in biotechnology 22, 559-565.

Settmacher, B., Bock, D., Saad, H., Gartner, S., Rheinheimer, C., Kohl, J., Bautsch, W., Klos, A., 1999. Modulation of C3a Activity: Internalization of the Human C3a Receptor and its Inhibition by C5a. The Journal of Immunology 162, 7409-7416.

Sharma, P., Lata, H., Arya, D.K., Kashyap, A.K., Kumar, H., Dua, M., Ali, A., Johri, A.K., 2013. Role of pilus proteins in adherence and invasion of Streptococcus agalactiae to the lung and cervical epithelial cells. J Biol Chem 288, 4023-4034.

Shimomura, Y., Okumura, K., Murayama, S.Y., Yagi, J., Ubukata, K., Kirikae, T., Miyoshi-Akiyama, T., 2011. Complete genome sequencing and analysis of a Lancefield group G Streptococcus dysgalactiae subsp. equisimilis strain causing streptococcal toxic shock syndrome (STSS). BMC Genomics 12, 17.

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

Siezen, R.J., de Vos, W.M., Leunissen, J.A., Dijkstra, B.W., 1991. Homology modelling and protein engineering strategy of subtilases, the family of subtilisin-like serine proteinases. Protein Eng 4, 719-737.

Sigh, J., Lindenstrøm, T., Buchmann, K., 2004. The parasitic ciliate Ichthyophthirius multifiliis induces expression of immune relevant genes in rainbow trout, Oncorhynchus mykiss (Walbaum). Journal of Fish Diseases 27, 409-417.

Silver, S., 1996. Bacterial resistances to toxic metal ions--a review. Gene 179, 9-19.

Smoot, L.M., Smoot, J.C., Graham, M.R., Somerville, G.A., Sturdevant, D.E., Migliaccio, C.A., Sylva, G.L., Musser, J.M., 2001a. Global differential gene expression in response to growth temperature alteration in group A Streptococcus. Proc Natl Acad Sci U S A 98, 10416-10421.

Smoot, L.M., Smoot, J.C., Graham, M.R., Somerville, G.A., Sturdevant, D.E., Migliaccio, C.A.L., Sylva, G.L., Musser, J.M., 2001b. Global differential gene expression in response to growth temperature alteration in group A Streptococcus. Proceedings of the National Academy of Sciences 98, 10416-10421.

Snyderman, R., Phillips, J., Mergenhagen, S.E., 1970. Polymorphonuclear leukocyte chemotactic activity in rabbit serum and Guinea pig serum treated with immune complexes: evidence for c5a as the major chemotactic factor. Infection and immunity 1, 521-525.

Snyderman, R., Pike, M.C., McCarley, D., Lang, L., 1975. Quantification of mouse macrophage chemotaxis in vitro: role of C5 for the production of chemotactic activity. Infection and immunity 11, 488-492.

Söderberg, J.J., von Pawel-Rammingen, U., Medicinsk, f., Umeå, u., Molekylärbiologi, 2008. The streptococcal protease IdeS modulates bacterial IgGFc binding and generates 1/2Fc fragments with the ability to prime polymorphonuclear leucocytes. Molecular immunology 45, 3347-3353.

St Geme, J.W., 3rd, Cutter, D., 1996. Influence of pili, fibrils, and capsule on in vitro adherence by Haemophilus influenzae type b. Mol Microbiol 21, 21-31.

St Geme, J.W., 3rd, Falkow, S., 1991. Loss of capsule expression by Haemophilus influenzae type b results in enhanced adherence to and invasion of human cells. Infect Immun 59, 1325-1333.

Stadtman, E.R., Berlett, B.S., Chock, P.B., 1990. Manganese-dependent disproportionation of hydrogen peroxide in bicarbonate buffer. Proc Natl Acad Sci U S A 87, 384-388.

Sternberg, M.J.E., Kelley, L.A., 2009. Protein structure prediction on the Web: a case study using the Phyre server. Nature Protocols 4, 363-371.

Stins, M.F., Badger, J., Sik Kim, K., 2001. Bacterial invasion and transcytosis in transfected human brain microvascular endothelial cells. Microb Pathog 30, 19-28.

Storz, G., Imlay, J.A., 1999. Oxidative stress. Curr Opin Microbiol 2, 188-194.

Svensson, M.D., Sjobring, U., Luo, F., Bessen, D.E., Sektionen för mikrobiologi, i.o.g.M.I.G., Department of Laboratory Medicine, L., Lund, U., Lunds, u., Division of Microbiology, I., Glycobiology, M.I.G., Institutionen för laboratoriemedicin, L., Faculty of, M., Medicin, 2002. Roles of the plasminogen activator streptokinase and the plasminogen-associated M protein in an experimental model for streptococcal impetigo. Microbiology (Reading, England) 148, 3933.

Talkington, D.F., Brown, B.G., Tharpe, J.A., Koenig, A., Russell, H., 1996. Protection of mice against fatal pneumococcal challenge by immunization with pneumococcal surface adhesin A (PsaA). Microbial pathogenesis 21, 17-22.

Tamura, F., Nakagawa, R., Akuta, T., Okamoto, S., Hamada, S., Maeda, H., Kawabata, S., Akaike, T., 2004. Proapoptotic effect of proteolytic activation of matrix metalloproteinases by Streptococcus pyogenes thiol proteinase (Streptococcus pyrogenic exotoxin B). Infect Immun 72, 4836-4847.

Tapp, J., Thollesson, M., Herrmann, B., 2003. Phylogenetic relationships and genotyping of the genus Streptococcus by sequence determination of the RNase P RNA gene, rnpB. Int J Syst Evol Microbiol 53, 1861-1871.

Tauber, M.G., Moser, B., 1999. Cytokines and chemokines in meningeal inflammation: biology and clinical implications. Clin Infect Dis 28, 1-11; quiz 12.

Telser, A., 2002. Molecular Biology of the Cell, 4th Edition. Shock 18, 289.

Terao, Y., Mori, Y., Yamaguchi, M., Shimizu, Y., Ooe, K., Hamada, S., Kawabata, S., 2008. Group A streptococcal cysteine protease degrades C3 (C3b) and contributes to evasion of innate immunity. The Journal of biological chemistry 283, 6253-6260.

Tettelin, H., Masignani, V., Cieslewicz, M.J., Donati, C., Medini, D., Ward, N.L., Angiuoli, S.V., Crabtree, J., Jones, A.L., Durkin, A.S., Deboy, R.T., Davidsen, T.M., Mora, M., Scarselli, M., Margarit y Ros, I., Peterson, J.D., Hauser, C.R., Sundaram, J.P., Nelson, W.C., Madupu, R., Brinkac, L.M., Dodson, R.J., Rosovitz, M.J., Sullivan, S.A., Daugherty, S.C., Haft, D.H., Selengut, J., Gwinn, M.L., Zhou, L., Zafar, N., Khouri, H., Radune, D., Dimitrov, G., Watkins, K., O'Connor, K.J., Smith, S., Utterback, T.R., White, O., Rubens, C.E., Grandi, G., Madoff, L.C., Kasper, D.L., Telford, J.L., Wessels, M.R., Rappuoli, R., Fraser, C.M., 2005. Genome analysis of multiple pathogenic isolates of Streptococcus agalactiae: implications for the microbial "pan-genome". Proc Natl Acad Sci U S A 102, 13950-13955.

Tettelin, H., Nelson, K.E., Paulsen, I.T., Eisen, J.A., Read, T.D., Peterson, S., Heidelberg, J., DeBoy, R.T., Haft, D.H., Dodson, R.J., Durkin, A.S., Gwinn, M., Kolonay, J.F., Nelson, W.C., Peterson, J.D., Umayam, L.A., White, O., Salzberg, S.L., Lewis, M.R., Radune, D., Holtzapple, E., Khouri, H., Wolf, A.M., Utterback, T.R., Hansen, C.L., McDonald, L.A., Feldblyum, T.V., Angiuoli, S., Dickinson, T., Hickey, E.K., Holt, I.E., Loftus, B.J., Yang, F., Smith, H.O., Venter, J.C., Dougherty, B.A., Morrison, D.A., Hollingshead, S.K., Fraser, C.M., 2001. Complete genome sequence of a virulent isolate of Streptococcus pneumoniae. Science 293, 498-506.

Thern, A., Stenberg, L., Dahlbäck, B., Lindahl, G., Division, V., Diagnostic, R., Lund, U., Department of Laboratory Medicine, M., Sektion, V., Klinisk kemi, M., Lunds, u., Institutionen för laboratoriemedicin, M., Department of Laboratory Medicine, L., Department of Clinical Sciences, L., Sektionen för medicinsk, m., Institutionen för kliniska vetenskaper, L., Clinical Chemistry, M., Diagnostisk radiologi, L., Institutionen för laboratoriemedicin, L., Faculty of, M., Medicin, Division of Medical, M., 1995. Ig-binding surface proteins of Streptococcus pyogenes also bind human C4b-binding protein (C4BP), a regulatory component of the complement system. Journal of immunology (Baltimore, Md. : 1950) 154, 375.

Thompson, R.A., Lachmann, P.J., 1970. Reactive lysis: the complement-mediated lysis of unsensitized cells. I. The characterization of the indicator factor and its identification as C7. The Journal of experimental medicine 131, 629-641.

Tordello, E.D., Vacca, I., Ram, S., Rappuoli, R., Serruto, D., 2014. Neisseria meningitidis NalP cleaves human complement C3, facilitating degradation of C3b and survival in human serum. Proceedings of the National Academy of Sciences 111, 427-432.

Tsao, N., Tsai, W.-H., Lin, Y.-S., Chuang, W.-J., Wang, C.-H., Kuo, C.-F., 2006. Streptococcal pyrogenic exotoxin B cleaves properdin and inhibits complement-mediated opsonophagocytosis. Biochemical and biophysical research communications 339, 779-784.

Uhlen, M., Guss, B., Nilsson, B., Gatenbeck, S., Philipson, L., Lindberg, M., 1984. Complete sequence of the staphylococcal gene encoding protein A. A gene evolved through multiple duplications. J Biol Chem 259, 1695-1702.

Unkmeir, A., Latsch, K., Dietrich, G., Wintermeyer, E., Schinke, B., Schwender, S., Kim, K.S., Eigenthaler, M., Frosch, M., 2002. Fibronectin mediates Opc-dependent internalization of Neisseria meningitidis in human brain microvascular endothelial cells. Mol Microbiol 46, 933-946.

Unnikrishnan, M., Cohen, J., Sriskandan, S., 1999. Growth-Phase-Dependent Expression of Virulence Factors in an M1T1 Clinical Isolate of Streptococcus pyogenes. Infection and immunity 67, 5495-5499.

Urban, C.F., Lourido, S., Zychlinsky, A., Molekylär Infektionsmedicin, S., Molekylärbiologi, Medicinska, f., Umeå, u., 2006. How do microbes evade neutrophil killing? Cellular microbiology 8, 1687-1696.

Van Beek, J., Bernaudin, M., Petit, E., Gasque, P., Nouvelot, A., MacKenzie, E.T., Fontaine, M., 2000. Expression of Receptors for Complement Anaphylatoxins C3a and C5a Following Permanent Focal Cerebral Ischemia in the Mouse. Experimental Neurology 161, 373-373.

Van Epps, D.E., Simpson, S.J., Johnson, R., 1993. Relationship of C5a receptor modulation to the functional responsiveness of human polymorphonuclear leukocytes to C5a. The Journal of Immunology 150, 246-252.

van Furth, A.M., Roord, J.J., van Furth, R., 1996. Roles of proinflammatory and anti-inflammatory cytokines in pathophysiology of bacterial meningitis and effect of adjunctive therapy. Infect Immun 64, 4883-4890.

van Sorge, N.M., Doran, K.S., 2012. Defense at the border: the blood-brain barrier versus bacterial foreigners. Future Microbiol 7, 383-394.

van Sorge, N.M., Zialcita, P.A., Browne, S.H., Quach, D., Guiney, D.G., Doran, K.S., 2011. Penetration and activation of brain endothelium by Salmonella enterica serovar Typhimurium. J Infect Dis 203, 401-405.

Vandesompele, J., De Preter, K., Pattyn, F., Poppe, B., Van Roy, N., De Paepe, A., Speleman, F., 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3, RESEARCH0034.

Vogel, U., Hammerschmidt, S., Frosch, M., 1996. Sialic acids of both the capsule and the sialylated lipooligosaccharide of Neisseria meningitis serogroup B are prerequisites for virulence of meningococci in the infant rat. Med Microbiol Immunol 185, 81-87.

von Heijne, G., 1983. Patterns of amino acids near signal-sequence cleavage sites. Eur J Biochem 133, 17-21.

Watanabe, Y., Todome, Y., Ohkuni, H., Sakurada, S., Ishikawa, T., Yutsudo, T., Fischetti, V.A., Zabriskie, J.B., 2002. Cysteine protease activity and histamine release from

the human mast cell line HMC-1 stimulated by recombinant streptococcal pyrogenic exotoxin B/streptococcal cysteine protease. Infection and immunity 70, 3944-3947.

Weeks, C.R., Ferretti, J.J., 1986. Nucleotide sequence of the type A streptococcal exotoxin (erythrogenic toxin) gene from Streptococcus pyogenes bacteriophage T12. Infect Immun 52, 144-150.

Weinstein, M.R., Willey, B.M., Borczyk, A., Low, D.E., Litt, M., Kertesz, D.A., Wyper, P., Rose, D., Coulter, M., McGeer, A., Facklam, R., Ostach, C., 1997. Invasive Infections Due to a Fish Pathogen, Streptococcus iniae. The New England Journal of Medicine 337, 589-594.

Wexler, D.E., Chenoweth, D.E., Cleary, P.P., 1985. Mechanism of Action of the Group A Streptococcal C5a Inactivator. Proceedings of the National Academy of Sciences of the United States of America 82, 8144-8148.

Wexler, D.E., Cleary, P.P., 1985. Purification and characteristics of the streptococcal chemotactic factor inactivator. Infect Immun 50, 757-764.

Wheelock, M.J., Johnson, K.R., 2003. Cadherin-mediated cellular signaling. Curr Opin Cell Biol 15, 509-514.

Wolburg, H., Lippoldt, A., 2002. Tight junctions of the blood-brain barrier: development, composition and regulation. Vascul Pharmacol 38, 323-337.

Woo, P.T.K., 1996. Protective immune response of fish to parasitic flagellates. Annual Review of Fish Diseases 6, 121-131.

Yamamoto, Y., Fukui, K., Koujin, N., Ohya, H., Kimura, K., Kamio, Y., 2004. Regulation of the Intracellular Free Iron Pool by Dpr Provides Oxygen Tolerance to Streptococcus mutans. Journal of Bacteriology 186, 5997-6002.

Yancey, K., Lazarova, Z. 2008. Complement System, In: Gaspari, A., Tyring, S. (Eds.) Clinical and Basic Immunodermatology. Springer London, 105-118.

Yancey, K.B., 1988. Biological properties of human C5a: selected in vitro and in vivo studies. Clinical and experimental immunology 71, 207-210.

Yang, Y., Kadim, M.I., Khoo, W.J., Zheng, Q., Setyawati, M.I., Shin, Y.-J., Lee, S.-C., Yuk, H.-G., 2014. Membrane lipid composition and stress/virulence related gene expression of Salmonella Enteritidis cells adapted to lactic acid and trisodium phosphate and their resistance to lethal heat and acid stress. International journal of food microbiology 191, 24.

Yano, T. 1997. 3 The Nonspecific Immune System: Humoral Defense, In: Elsevier Science & Technology, 105-157.

Yao, J., Harvath, L., Gilbert, D.L., Colton, C.A., 1990. Chemotaxis by a CNS macrophage, the microglia. Journal of neuroscience research 27, 36-42.

Zhang, B.C., Zhang, J., Sun, L., 2014. Streptococcus iniae SF1: complete genome sequence, proteomic profile, and immunoprotective antigens. PLoS One 9, e91324.

Zhang, W.G., Khan, A.N., Kim, K.J., Stins, M., Kim, K.S., 2002. Transforming growth factor-beta increases Escherichia coli K1 adherence, invasion, and transcytosis in human brain microvascular endothelial cells. Cell Tissue Res 309, 281-286.

Zhu, Y., Thangamani, S., Ho, B., Ding, J.L., 2005. The ancient origin of the complement system. EMBO J 24, 382-394.

Zinkernagel, A.S., Timmer, A.M., Pence, M.A., Locke, J.B., Buchanan, J.T., Turner, C.E., Mishalian, I., Sriskandan, S., Hanski, E., Nizet, V., 2008. The IL-8 protease SpyCEP/ScpC of group A Streptococcus promotes resistance to neutrophil killing. Cell Host Microbe 4, 170-178.

Zinser, E.R., Kolter, R., 2004. Escherichia coli evolution during stationary phase. Research in microbiology 155, 328-336.

Zipfel, P.F., Skerka, C., Hellwage, J., Jokiranta, S.T., Meri, S., Brade, V., Kraiczy, P., Noris, M., Remuzzi, G., 2002. Factor H family proteins: on complement, microbes and human diseases. Biochemical Society transactions 30, 971.

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

Zwirner, J., Fayyazi, A., Götze, O., 1999. Expression of the anaphylatoxin C5a receptor in non-myeloid cells. Molecular immunology 36, 877-884.

Appendix A

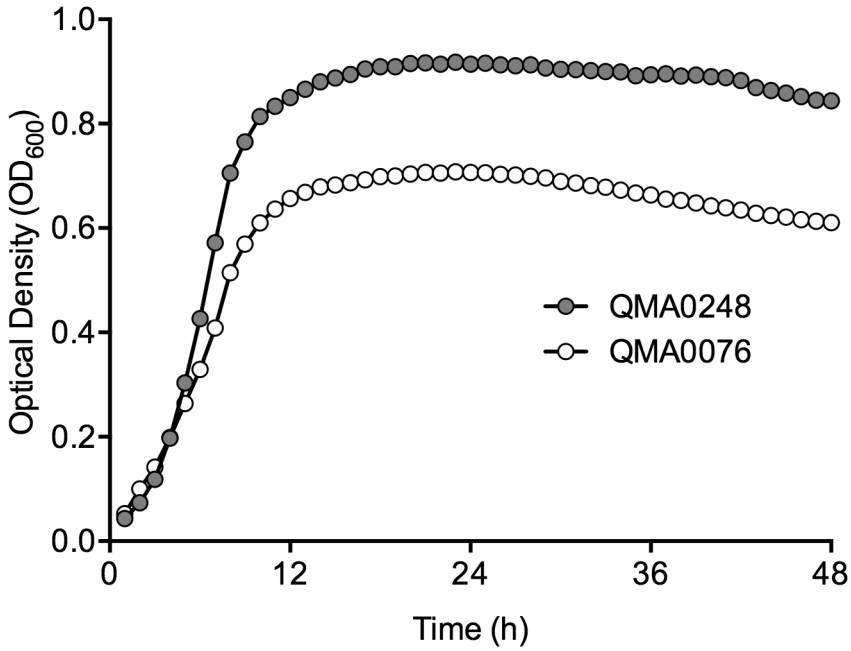

Figure 19. Growth of S. iniae QMA0248 and S. iniae QMA0076 in Todd-Hewit Broth

Table 2. Sampling time points of S.iniae strains grown in Todd-Hewit Broth

Strain	Time cells harvested		
	Mid-exponential	Late-exponential	Stationary
QMA0076	6 h 00 min	9 h 00 min	24 h 0 min
QMA0248	5 h 30 min	8 h 50 min	24 h 0 min

Appendix B

Table 3. Primer sequences for fusion PCR to generate knock-out insertion

No.	Primers	Sequences 5'-3'	Length	GC%	Tm	Amplicon (bp)
1	F1-scpi	TGGGGTTGTTAACGTTGGTCT	21	47.62	59.79	1058
2	R1-scpi	ATTCAAATATATCCTCCTCAATATATTCCTCCAATAGAT	39	28	69.4	
3	F3-scpi	AAACTATTTAAATAACAGATTATCAAAAAAGAATGTTCGCCT	42	24	69.2	1058
4	R3-scpi	TGCTGAGAGTTATGGCGCA	20	50	60.04	
5	F-all-scpi	AGCAACACCTTGGTTAGCAGT	21	47.62	60.13	2544
6	R-all-scpi	TTGCAGCTTACTACCGCTCT	20	50	59.10	
7	F1-cppa	ACTTTCCATCAAGGCATCACG	21	48	59.5	917

8	R1-cppa	ATTCAAATATATCCTCCTCCATTACCTCTTTTTGACCTCA	40	35	72.8	
9	F3-cppa	AAACTATTTAAATAACAGATTGGTTTCCTCCTAACCTCT	39	31	70.6	1016
10	R3-cppa	CTGAATAGGGCTTGCGCTGA	20	55	60.5	
11	F-all-cppa	AGCCGCTTCTTGCTTATTGTTT	22	40.91	59.44	2428
12	R-all-cppa	CACTGACAATGCCGGCAAAA	20	50	58.4	
13	F2-aad9	GAGGAGGATATATTTGAATACATACG	26	35	61.7	753
14	R2-aad9	AATCTGTTATTTAAATAGTTTATAGTTA	28	14	55.4	