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Characterization of virulence factors in the aquatic pathogen Streptococcus iniae

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Characterization of virulence factors in the aquatic pathogen *Streptococcus iniae*

A dissertation submitted in partial satisfaction of the
Requirements for the degree Doctor of Philosophy

in

Marine Biology

by

Jeffrey Brian Locke

Committee in Charge:

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2008

The Dissertation of Jeffrey Brian Locke is approved, and it is acceptable in quality and form for publication on microfilm:

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Chair

University of California, San Diego

2008

DEDICATION

To my family and friends

TABLE OF CONTENTS

LIST OF FIGURES

Chapter II

Chapter III

Chapter IV

Chapter V

LIST OF TABLES

LIST OF COMMON ABBREVIATIONS

AMP	antimicrobial peptide
BLAST	basic local alignment search tool
bp	base pair
cat	chloramphenicol acetyltransferase
cepI	*S. iniae* cell envelope proteinase, IL-8 protease gene
CFU	colony-forming units
CLC	carp macrophage cell line
Cm	chloramphenicol
cpsD	*S. iniae* capsule biosynthesis gene
DMEM	Dulbeco's modified Eagle's medium
Erm	erythromycin
FBS	fetal bovine serum
GAS	*S. pyogenes*, group A *Streptococcus*
GBS	*S. agalactiae*, group B *Streptococcus*
HSB	hybrid striped bass (*Morone chrysops* x *M. saxatilis*)
IM	intramuscular
IP	intraperitoneal
K288	virulent *S. iniae* strain
kDa	kilodaltons
LA	Luria-Bertani agar
LB	Luria-Bertani broth
LD	lethal dose
µg	microgram
µl	microliter
ml	milliliter
Mga	GAS transcriptional regulatory protein
Mgx	*S. iniae* putative Mga-like regulatory protein
MOI	multiplicity of infection
OD	optical density

ORF	open reading frame
PBS	phosphate buffered saline
pgmA	*S. iniae* phosphoglucomutase gene
RPS	relative percent survival
sagA	structural gene of the *S. iniae* SLS cytolysin
scpI	*S. iniae* C5a peptidase-like gene
simA, B	*S. iniae* M-like protein genes
SiM	*S. iniae* M-like protein
S. iniae	*Streptococcus iniae*
SLS	streptolysin S
Spect	spectinomycin
THA	Todd-Hewitt agar
THB	Todd-Hewitt broth
WBE27	white bass epithelial cell line
WT	wild-type

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Chapter 2 is a full reprint of the publication: Locke, J.B., Colvin, K.M., Vicknair, M.R., Varki, N., Nizet, V., and J.T. Buchanan. 2007. The *Streptococcus iniae* β-hemolysin streptolysin S is a virulence factor in fish infection. *Diseases of Aquatic Organisms*. 76(1): 17–26, with permission from all coauthors.

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ABSTRACT OF THE DISSERTATION

Characterization of virulence factors in the aquatic pathogen *Streptococcus iniae*

by

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Doctor of Philosophy in Marine Biology

University of California, San Diego, 2008

Professor Douglas Bartlett, Chair

Streptococcus iniae has emerged as a leading pathogen of intensive finfish aquaculture operations worldwide, though an understanding of its virulence mechanisms and effective therapeutic strategies are lacking. The roles of four putative *S. iniae* virulence factors: Streptolysin S (SLS), capsular polysaccharide, M-like protein, and C5a peptidase, were investigated through targeted allelic exchange mutagenesis coupled with *in vitro* and *in vivo* models of bacterial pathogenesis. Highly attenuated mutants were then evaluated as live vaccine candidates.

SLS is a potent, broad-spectrum, secreted cytolysin first characterized in group A *Streptococcus*. Allelic replacement of the *sagA* gene in the *S. iniae* SLS operon

generated a mutant that was significantly less cytotoxic to a variety of fish cell types. Reduced cytotoxicity *in vivo* towards brain cells in particular may account for the mutant's high level of attenuation in a hybrid striped bass (HSB) challenge model.

Capsular polysaccharide serves as a protective extracellular coating in many pathogenic streptococci. Targeted allelic replacement of the *S. iniae cpsD* capsule biosynthesis gene generated a mutant that was completely attenuated in HSB even at a dose 1,000X the lethal WT dose, likely due to its high susceptibility to phagocytic clearance, as demonstrated with a fish macrophage cell line.

Through pyrosequencing of the *S. iniae* genome we identified gene homologues to classical surface-anchored streptococcal virulence factors: M-like protein (*simA*) and C5a peptidase (*scpI*). Analysis of the corresponding allelic mutants in HSB and zebrafish revealed that only *simA* contributes significantly to *S. iniae* pathogenesis. *In vitro* cell-based analyses indicated that SiMA, like other M family proteins, contributes to adherence and invasion and provides resistance to phagocytic killing.

The immunoprotective capacity of the Δ*sagA*, Δ*cpsD*, and Δ*simA* attenuated mutant strains was compared with a killed bacterin vaccine in HSB through injection and immersion delivery. The Δ*cpsD*, Δ*simA*, and bacterin vaccines met vaccination safety criteria and were effective in generating high levels of immune protection through injection, however the Δ*simA* mutant was the only vaccine candidate to provide 100% protection through both injection and immersion delivery.

Characterization of these four genes has contributed significantly to our understanding of *S. iniae* virulence mechanisms and demonstrates the application of pathogenesis research to vaccine development.

Chapter I

Introduction and background

Introduction

Since its isolation from an Amazon freshwater dolphin (*Inia geoffrensis*) in the 1970s (89), *Streptococcus iniae* has emerged as a leading finfish pathogen in aquaculture operations worldwide, resulting in over $100M in annual loses (101). As the global demand for seafood increases and natural fisheries continue to decline, the prevalence of aquaculture and associated pathogens such as *S. iniae* will likely increase. Understanding how this pathogen causes disease is critical in the development of novel vaccines, therapeutics, and finfish husbandry practices to reduce *S. iniae* outbreaks. Taking advantage of over a century of research on the closely related human pathogens group A and B *Streptococcus* (GAS, GBS), the elucidation of *S. iniae* virulence mechanisms has progressed rapidly. Just within the last five years, the use of advanced sequencing techniques and targeted mutagenesis approaches has led to identification of specific genes encoding virulence factors involved with many key stages of *S. iniae* disease pathogenesis. With the first *S. iniae* genome sequencing project near completion, understanding of the genetic underpinning of virulence will be further accelerated. Presented here is a review of the current knowledge of *S. iniae* virulence mechanisms, their genetic determinants, and the corresponding application towards the development of live attenuated vaccines for use in aquaculture.

Background

Isolation, phylogenetics, typing, and classifications

The first published report of a streptococcal finfish infection documented an outbreak in trout that occurred in Japan 50 years ago (46). *S. iniae* was not officially identified and characterized until 1976, following its isolation from an Amazon freshwater dolphin skin abscess (89). Around this era a number of streptococcal outbreaks occurred in Asia (52, 79), the US (91), and the Middle East (28). These strains (28, 30), and some of the strains associated with early Japanese outbreaks (52, 79, 80) were later suggested to be *S. iniae* (51).

S. iniae falls into a general category of non-typable Lancefield group β-hemolytic streptococci (59) and elucidating the geographical origin and global dissemination of *S. iniae* was complicated by deficiencies in naming conventions, lack of sequencing technologies, and limited phenotypic characterization assays. 16S-based phylogenetic analyses place *S. iniae* close to other streptococcal pathogens of humans and animals (60, 114). Genomic restriction fragment analysis of diverse host and geographical panels of *S. iniae* isolates has shown common profiles between virulent fish and human strains (36, 39, 81, 108), though multiple PFGE (pulsed field gel electorphoresis) patterns have been identified among human isolates (36, 60).

Currently there are two established *S. iniae* serotypes. The ATCC 29178 type strain first characterized by Pier and Madin (89) is representative of Serotype I isolates. Serotype II was first identified as the type strain (ATCC 29177) isolated from another dolphin case of “golf ball disease” (90). A biochemical assay measuring arginine dihydrolase (ADH) activity has been used to distinguish between serotypes (Serotype I is

positive) (4), though proposed hyper-encapsulation of Serotype II may represent the most significant functional difference between the two types (7).

Host range

To date *S. iniae* infections have been documented in over 30 species of finfish (1, 51, 94), many of which are economically important, including trout (29, 52), tilapia (28, 52, 87), salmon (29), yellowtail (79), barramundi (14), ayu (52), European seabass (114), Japanese flounder (80), red drum (114) and hybrid striped bass (HSB) (104). Fish raised in intensive aquaculture operations and subject to environmental stressors (i.e. suboptimal temperature, poor water quality, crowding, handling, etc.) are most prone to *S. iniae* infection (12, 82, 88, 100, 111). Mortality from outbreaks occurring under these conditions can be as high as 75% (87). Wild fish populations located proximally (22, 114) and distally (37) to aquaculture operations have proven susceptible to *S. iniae* infection as well. Some fish do demonstrate inherent resistance to *S. iniae* infection, including carp (29), channel catfish (88), and sea and one-spot bream (111). Aside from finfish, freshwater dolphins (11, 89, 90) and humans (108) have been the most common host for *S. iniae*. There are reports of *S. iniae* isolated from bullfrogs in the US (72) and from flying foxes in Australia (1), though its significance in these species is unknown.

Disease pathogenesis

S. iniae infection in finfish is typically characterized by clinical symptoms such as exopthalmia, ulcers, lethargy, loss of orientation, dark skin coloration, and anorexia (28, 51). Invasive *S. iniae* isolates can cause sepsis and systemic spread of the bacteria

through the bloodstream and major organs, often leading to significant aggregation in the brain (18) where *S. iniae* causes fatal meningoencephalitis (29). Type I isolates tend to cause chronic infections whereas the potentially more encapsulated type II strains typically cause more acute, systemic infections (58, 113).

In contrast to finfish infections, *S. iniae* does not cause systemic, lethal disease in freshwater dolphins. *S. iniae* has been recovered from cutaneous and subcutaneous infections sites on dolphins (11, 89, 90), however, definitive proof for *S. iniae* as the etiological agent of disease through examination of Koch's postulates has not been completed (10).

Human cases of *S. iniae* infection typically occur in elderly or immunocompromised people, and are especially associated with injuries while handling live or fresh infected fish (1, 108). Similar to *S. iniae* infections in dolphins, human infections tend to be limited to localized soft tissue infections (as opposed to systemic, lethal infections in teleosts) of the upper limbs and are easily treated with common antibiotics. The first documented human case of *S. iniae* infection in North America occurred in 1991, and was followed by nine additional cases in Toronto, Canada in the mid-1990s (108). Human cases have also been reported in China (60), Taiwan (105), and Singapore (56). Asian descent is a common trend in the majority of invasive human cases, but it is unknown whether this is due to inherent differences in immunity or because of cultural fish preparation habits which lend themselves to a higher incidence of infection (1).

Transmission

Environmental survival and transmission mechanisms of *S. iniae* relevant to fish infection are not completely understood. Characterization of bacterial dissemination following oral or immersion *S. iniae* challenge in Japanese flounder suggests that entry through abrasions on the skin or fins is a much more likely route than entry through the gills, nares, eyes, or GI tract (86). In flounder, oral infection was only achieved at high inoculation doses (85), indicating that oral transmission in natural outbreaks may be due to cannibalism of infected fish, where *S. iniae* can reach levels as high as 10^9 colony-forming units (CFU) per gram of tissue in the spleen, kidney, and brain or an equivalent concentration per milliliter of blood (18, 66, 86, 102). High density rearing conditions, where cannibalism or fecal-oral transmission may occur, are also likely scenarios where close contact between fish and the rearing tank itself may damage the integrity of the epidermal and protective mucosal layers and predispose fish to colonization by *S. iniae* (100). This transmission hypothesis is supported by data which show that experimental infection by immersion is often aided through manual injury to the fish prior to challenge with *S. iniae* (83).

The existence of *S. iniae* in the aquatic environment is not well understood. While some studies have failed to isolate *S. iniae* surrounding sites of infection (88) others have found it year around in the water and sediments surrounding aquaculture operations where it predominantly causes infection in summer months (84), a period when warm water favors *S. iniae* and increases fish stress and susceptibility to disease. Aside from therapeutic intervention, good husbandry practices are the most effective way to reduce the risk of *S. iniae* infection.

Identification of virulence factors and associated genes

Prior to genomic sequencing efforts, transposon mutagenesis (Tn*916, 917, 4001*) was employed to identify potential *S. iniae* virulence genes through random chromosomal insertions. These early mutagenesis studies identified two of the most import virulence determinants to date: streptolysin S (SLS) (40) and capsular polysaccharide (77), as well as some less traditional virulence factors such as phosphoglucomutase (18). Subsequent targeted mutagenesis (40, 65, 66, 68) or complementation techniques (18) were then used to confirm the respective roles of these virulence determinants. While a number of genes have been identified through transposon mutagenesis alone, without complementation or precise targeted mutagenesis data it is impossible to conclusively link them to observed transposon mutant attenuation, and only genes meeting the forementioned criteria are included in Table 1.1.

With the advent of cheaper and more high-throughput sequencing technologies, such as pyrosequencing (454 Life Sciences) (69), putative virulence genes have been be rapidly identified (based on homology to known virulence factors in streptococcal pathogens such as GAS and GBS) and characterized directly through targeted mutagenesis (64, 112). The first official *S. iniae* genome sequencing project (human isolate, strain 9117) at the Baylor College of Medicine Human Genome Sequencing Center (www.hgsc.bcm.tmc.edu) is near completion and will greatly help to broaden our knowledge of the genetic basis for *S. iniae* virulence and help to inform future mutagenesis-based studies.

Tools used to characterize virulence factors

A number of *in vitro* and *ex vivo* assays have been utilized to help deduce contributions of various *S. iniae* virulence factors. Assays have been designed to measure very specific virulence aspects such as sensitivity to host antimicrobial peptides (17, 18, 64, 65, 76), lysozyme (76), and oxidative killing (17, 65). More recently, real time PCR has been utilized to determine the effect of regulatory proteins on the expression of *S. iniae* virulence factors (8, 9). A number of cell-based virulence assays have also been utilized from both established cell lines and also from primary isolation of cells from fish and mammalian models. Fish cell lines including white bass epithelial cells (WBE27, ATCC # CRL-2773) (17, 64-66, 76, 98), carp macrophages (CLC, European Collection of Cell Cultures # 95070628) (17, 64-66), rainbow trout macrophages (RTS-11) (41, 113), salmonid embryonic cells (CHSE-214) (113), rainbow trout gonad cells (RTG-2) (113), and zebrafish hepatocytes (ZFL, ATCC # CRL-2643) (78) as well as primary cells derived from fish including epithelial cells (35), peritoneal macrophages (5, 54, 102, 113), pronephros phagocytes (PN) (3, 113), blood leukocytes (PBL) (113), nonspecific cytotoxic cells (NCC) (31, 106), and red blood cells (64) have all been important tools in measuring cell-based virulence properties such as adherence, invasion, cytotoxicity, and various aspects of the phagocytic killing process. Mammalian derived cells and cell lines have also been utilized to study these aspects of *S. iniae* virulence, including murine macrophages (RAW) (77), human epithelial cells (Hep-2) (39), human brain microvascular endothelial cells (BMEC) (39), and sheep red blood cells (17, 40). Fish whole blood (17, 18, 65, 66, 76, 113) and serum (6, 17, 102) and human whole blood (8, 36, 40, 77, 112, 113) and neutrophils (112) have been used for *ex*

vivo measurement of resistance to complement and other innate host defense mechanisms.

Experimental reproduction of *S. iniae* disease *in vivo* has been achieved through the development of animal models and is the ultimate deciding factor in assessing virulence mechanisms. Animal models of *S. iniae* pathogenesis include: HSB (18, 34), tilapia (29), red-tail black and rainbow sharks (94), barramundi (13, 14), zebrafish (83), trout (29), Japanese flounder (85), red drum (97), mice (39). Intraperitoneal (IP) and intramuscular (IM) delivery of bacteria in model challenge systems are the norm for *S. iniae* due to the ability to closely control dosage and achieve reproducibility. However, injections likely do not as closely mimic likely natural transmission scenarios. Immersion models are a closer replication of aquatic bacterial transmission, but are not as reproducible and often times healthy fish are highly resistant to immersion-based infection. Work with barramundi (13), tilapia (88), Japanese flounder (85, 86) has demonstrated efficacy of injection, immersion, and oral delivery of *S. iniae*. HSB and tilapia have shown susceptibility to *S. iniae* challenge through delivery to the gills (75) and nares (33, 34). Perhaps the most realistic *S. iniae* infection model employs infection through cohabitation of healthy fish with infected fish (100).

Virulence mechanisms and associated genes

Colonization and adherence

The first stage in pathogenesis involves colonization of the host, and *S. iniae* has demonstrated an ability to adhere to fish (65) and human epithelial cells (39). Interestingly, a dramatic increase in the adherence of a capsule mutant Δ*cpsD* has been

reported (65) (Table 1.1). This phenomenon could be a function of stronger charge interaction with host cells or may be attributed the exposure of surface-anchored cellular adhesion proteins otherwise concealed by the capsular polysaccharide layer. It has been shown that *S. pneumoniae* capsule levels are reduced upon adherence and invasion of host cells (43). A role has been demonstrated for the M-like protein, SiM, in adherence to a white bass epithelial cell line (64). In contrast to GAS (92) and GBS (21), our C5a peptidase-like protein knockout mutant (Δ*scpI*) did not show a reduced ability to adhere to fish epithelial cells (data not published).

Invasion and dissemination

Once colonization has occurred, *S. iniae* must invade host cells in order to disseminate throughout the host. This process also can be a strategy for intracellular evasion of the host immune system. Similarly to the trends seen in measuring adherence, an increase in invasiveness towards epithelial cells with bacteria lacking capsule has been observed (65), as has a decrease in invasiveness with *S. iniae* lacking M-like protein (64). Both virulent (9117) and commensal (9066) strains of *S. iniae* have demonstrated the ability to invade human epithelial cells, though the commensal isolates do so at significantly higher levels (39). Recent work using cultured primary trout epithelial cell layers revealed a transcytotic mechanism of passage through the monolayers, a property likely involved in *S. iniae* virulence (35). An *S. iniae* plasmin(ogen)-binding α-enolase surface protein may be one such factor contributing to tissue invasion and dissemination (50), though a confirmatory demonstration, through mutagenesis, of the role of this protein in *S. iniae* virulence has not been completed.

Table 1.1 Established *S. iniae* virulence factors and associated genes

Virulence factor	Gene(s) analyzed	Strain(s) analyzed	Proposed gene function(s)	Proposed role(s) of virulence factor	Mutagenesis technique	*In vivo* model(s)	Ref.
M-like protein (SiM)	*simA*	K288	binds host cells and fibrinogen	adherence/invasion, and resistance to phagocytic killing	allelic replacement	HSB zebrafish	(64)
capsular polysaccharide	*cpsD*	K288, 94-426	chain length and export	resistance to phagocytosis	allelic replacement	HSB	(65)
	cpsA	9117	chain length determination		targeted insertion	zebrafish	(68)
	cpsY	9117	capsule regulation		targeted deletion	zebrafish	(68)
phospholgucomutase	*pgmA*	K288	sugar metabolism	cell wall rigidity, resistance to AMPs	transposon insertion	HSB	(18)
peptidoglycan deacetylase	*pdi*	K288	peptidoglycan acetyl modifications	protection against lysozyme killing	allelic replacement	HSB	(76)
cell envelope proteinase	*cepI*	K288	IL-8-cleaving cysteine protease	inactivation of host immune signal	targeted insertion	mouse	(112
Streptolysin S	*sagA*	K288	SLS structural peptide	host cell injury	allelic replacement	HSB	(66)
	sagB	9117	SLS modification protein		allelic replacement	mouse	(40)
SivR/S	*sivR/S*	9117	two-component transcriptional regulation system	virulence gene regulation	allelic replacement	mouse	(9)

* Criteria for established virulence factor genes included *in vivo* attenuation of a targeted mutant or a transposon mutant with complementation data.

Host cell injury

One strategy implicated in dissemination and immune hindrance involves pathogen-mediated damage of host tissues. Early studies demonstrated general cytotoxicity of *S. iniae* towards human microvascular endothelial cells (BMEC) (39). The secreted cytolysin responsible for the characteristic zone of β-hemolysis around *S. iniae* colonies growing on blood agar plates was also implicated in such a virulence role (40, 66). Cytotoxicity assays demonstrated broad cytolytic activity of WT *S. iniae* against epithelial, leukocytic and blood cells compared to an isogenic SLS-deficient knockout strain (Δ*sagA*) (66) (Table 1.1). SLS has been confirmed to play a role in virulence using IP challenge of HSB (66), though we found that extended challenge duration revealed delayed killing kinetics and that the SLS mutant could colonize fish asymptomatically for months before causing a lethal infection (data not published). A common hematological observation during *S. iniae* infection is a reduction in circulating red blood cells (38, 74), but whether active *in vivo* hemolysis attributed to SLS contributes to this phenomena is unknown. A subcutaneous mouse infection model also revealed attenuation of an SLS-deficient mutant (Δ*sagB*) in lesion formation (40) (Table 1.1), suggesting that this virulence factor may be involved in human soft tissue infections. We have observed an increase in hemolysis in the Δ*cpsD* capsule mutant though the nature and potential *in vivo* function of such an effect is unknown and the mutant is still overall highly attenuated (data not published). Another putative cytotoxic virulence determinant identified in *S. iniae* is CAMP factor (93). The *cfi* gene encoding CAMP has recently been identified (9), but has yet to be a proven contributor to *S. iniae* virulence.

Immune evasion - Resistance to phagocytosis

Once established within the host, *S. iniae* must employ mechanisms to avoid immune clearance. *S. iniae* produces small extracellular products which provide a chemotactic signal for host phagocytes involved in the inflammatory response (54) and whole killed *S. iniae* cells have been shown to trigger the release of stimulatory signals to nonspecific cytotoxic cells (31). To avoid phagocytic clearance, one of the most important features that *S. iniae* possesses is its outer capsular polysaccharide coating. While lack of capsule is thought to aid in colonization and invasion of host tissues, encapsulation is necessary for survival in the bloodstream and overall virulence.

Early work with unclassified β-hemolytic streptococci isolated from rainbow trout in Japan showed the presence of extracellular capsule and its relative importance in fish virulence (110) though capsule was not officially identified in *S. iniae* till years later (7). Screening of *S. iniae* transposon mutant libraries in HSB (18) and zebrafish (77) supported earlier studies and revealed that putative capsule mutants represented a disproportionately high number of the attenuated mutants. This virulence trend was also supported in a virulence screen of a panel of naturally capsule-deficient *S. iniae* strains in Japanese flounder (49). Transposon mutants mapped to capsule genes *cpsH*, *cpsI*, *cpsM* and ORF 276 and genes putatively affecting capsule production (*citG*, *mtlA*, sensor histidine kinase, CBS domain containing protein, and adenylosuccinate synthase) all showed high levels of attenuation in a Japanese flounder challenge model (102), though without complementation data it is impossible to definitively link disruption of these genes to the observed virulence attenuation.

Analysis of targeted mutants confirmed that capsule was a key virulence factor (65, 68). Allelic replacement of the *cpsD* gene of the capsule biosynthesis operon resulted in complete attenuation even at a dose of 10^8 CFU in an HSB IP challenge model (65), while targeted mutations in the *cpsA* and *cpsY* genes generated mutants somewhat less attenuated when evaluated in a zebrafish IM challenge model (68) (Table 1.1). Cell-based *in vitro* assays with the attenuated Δ*cpsD* mutant conclusively demonstrated an antiphagocytic role for *S. iniae* capsule by showing a dramatic increase in phagocytic uptake of the mutant over the isogenic WT strain by fish macrophages (65).

Supporting the importance of capsule as a critical virulence factor, deficiencies in its production are thought to be a primary factor in determining whether a strain is virulent or commensal (77). Strain 9066, a commensal isolate, possesses a naturally occurring deletion of a region of its capsule biosynthesis operon (68). The original *S. iniae* ATCC 29178 dolphin skin abscess isolate (Serotype I) has been shown to be nonpathogenic in fish (17, 88) and mammalian (39, 89) infection models. This isolate also shares traits (i.e. increased phagocytic uptake, increased adherence to epithelial cells, decreased survival in whole blood, etc.) with capsule-deficient *S. iniae* strains (17), though capsule deficiency has yet to be proven.

Immune evasion - Resistance to phagocytic killing

In addition to capsular polysaccharide, pathogenic streptococci have a number of surface proteins which help the bacteria to resist phagocytic clearance, most notably M family proteins. The recently identified M-like protein (SiM) likely contributes to evasion of host immune defenses (5, 64). The ability of SiM to bind fibrinogen (5) may

function as an antiphagocytic feature that may help account for the *in vivo* attenuation seen in a SiM knockout strain (Δ*simA*) in HSB and zebrafish infection models (64) (Table 1.1). WT bacteria incubated with fibrinogen and then exposed to peritoneal macrophages from barramundi demonstrated a reduction in respiratory burst activity, indicative of reduced phagocytic killing (5). Incubation of the Δ*simA* allelic replacement mutant with a carp macrophage cell line revealed significantly diminished survival compared with the isogenic WT strain (64), supporting a phagocytic survival role for SiM. Capsular polysaccharide has demonstrated a similar phagocytic resistance function as seen in the increased generation of reactive oxygen species by peritoneal macrophages with intracellular capsule-deficient transposon mutants as opposed to the WT parental strain (102). Similarly, the avirulent type strain ATCC 29178 is sensitive to hydrogen peroxide killing assay (17).

Earlier phenotypic observations showed that *S. iniae* has the ability to bind the Fc region of Ig when grown in the presence of fish serum (6); further research is needed to determine if this antiphagocytic capacity, common to many streptococcal M-like proteins, should be attributed to SiM or if it is a function of an uncharacterized surface Fc binding protein. While many streptococcal M family proteins function in a broader capacity through antigenic variation, two independent sequencing efforts of diverse panels of *S. iniae* isolates have only succeeded in identifying two alleles of the *sim* gene: *simA* and *simB* (5, 64). The only other significant *sim* gene variation found to date involves a naturally occurring 40 bp insertion duplication frameshift mutation, splitting the *simA* gene into two putative ORFs of unknown function (64). This natural SiM mutant strain (02161A) was also attenuated in HSB infection models (64).

Immune evasion - Survival within immune cells

S. iniae also has strategies for survival within host immune cells. One theory proposes a "Trojan horse" strategy used by *S. iniae* to evade immune recognition and disseminate throughout the host (113). Type II strains (those responsible for more acute and systemic infections) have enhanced capacity to survive within macrophages and promote their apoptosis, both strategies may aid in dissemination and survival within the host (113). By surviving within peripheral blood leukocytes, including macrophages, *S. iniae* may more easily achieve systemic spread within the host and it is hypothesized that this hijacking of migrating macrophages may be the major means that *S. iniae* finds its way into the brain from the blood system, so causing fatal meningeal infections (113). We argue that while intracellular survival within host immune cells is likely a key factor in systemic spread, *S. iniae* would not benefit from complete phagocytic uptake and relies on its capsule to resist phagocytosis for much of the infectious population.

Immune evasion - Resistance to antimicrobial peptides and enzymes

Antimicrobial peptides (AMPs) and enzymes represent another class of host defense to which *S. iniae* has developed resistance mechanisms. Cell wall rigidity attributed to phosphoglucomutase (Pgm) activity affords the bacteria resistance against cationic AMPs such as HSB-derived moronecidin (61), as demonstrated by the increased AMP sensitivity of a Δ*pgmA* transposon mutant (18). Resistance to AMPs does not always translate into increased virulence. Reduction in surface capsular polysaccharide levels either through induced mutation (65) or as found in putative naturally occurring putative capsule mutants (17) results in increased AMP resistance, though virulence is

severely attenuated. Protection against host enzymes which target components of the cell wall is another defensive strategy of *S. iniae*. Allelic replacement of a surface-anchored peptidoglycan deacetylase (Pdi) resulted in increased sensitivity to lysozyme and whole blood killing and an overall decrease in virulence (Table 1.1) (76).

Immune evasion – Inactivation of host immune signaling molecules

Another virulence strategy for immune evasion involves inactivation of host immune signals. A cell envelope proteinase (CepI) in the *S. iniae* genome homologous to the GAS SpyCEP/scpC IL-8 protease was recently identified (44). A *cepI*-deficient targeted insertion mutant demonstrated reduced IL-8 cleavage, increased susceptibility to killing by human neutrophils, and reduced lesion size in a subcutaneous mouse infection model (112) (Table 1.1). Another target of streptococcal pathogens is the complement system chemoattractant C5a. Allelic replacement of the *S. iniae* C5a peptidase-like protein gene (*scpI*) however failed to produce attenuation in HSB or zebrafish models of streptococcal pathogenesis (64). Further analyses are required to see whether the ScpI retains proteolytic activity against C5a. *S. iniae* may have adopted alternative complement inactivation strategies, a hypothesis supported by the presence of a putative C3 proteinase in our analysis of genomic pyrosequence data (data not published).

Virulence gene regulation

While individual virulence factors contribute to various facets of pathogenesis, their expression levels must be tightly controlled order to optimize virulence as *S. iniae* encounters diverse host environments during the infectious process. Other streptococcal

pathogens are known to possess a number of virulence gene regulators critical to the pathogen's success. Regulation of capsular polysaccharide in *S. iniae*, one of the most critical virulence determinants, may occur in part via the *cpsY* gene of the capsule operon (68) which has high homology to the GBS *cpsY* gene that is thought to play a role in regulation of capsule biosynthesis (57). More conclusively, *S. iniae* capsule regulation has been shown to occur through a two-component (sensor histidine kinase coupled with a DNA response regulator) *sivS*/*R* regulatory system (8, 9) which shares homology to two-component systems implicated in the regulation of virulence genes (though not capsule) in GAS (103). Real time PCR analysis revealed that the 9117Δ*siv* allelic replacement mutant had a 70% reduction in transcription of the *cpsA* capsule biosynthesis gene (first gene in the capsule operon), corroborating phenotypic observation of reduced extracellular capsular polysaccharide in the Δ*siv* mutant strain (8). Further work revealed that the *sivR*/*S* system also regulates expression of additional virulence-associated genes, such as CAMP factor (*cfi*) and the streptolysin S precursor (*sagA*) (9). An untranslated RNA sequence including the GAS *sagA* gene has demonstrated virulence regulatory capabilities (62), but whether such a regulatory mechanism exists in *S. iniae* is unknown.

Another putative regulatory element found in *S. iniae* is the Mga-like Mgx protein. Mga (multiple gene regulator of group A *Streptococcus*) is a key virulence gene regulator of M family and C5a peptidase genes clustered within the Mga locus as well as virulence genes located distally in the chromosome (45). In *S. iniae*, the *mgx* gene is located adjacent to the divergently transcribed M-like protein gene *sim* (5, 64) and distally from the C5a peptidase-like protein gene *scpI* (64). *S. iniae* appears to have gone through an *mgx* gene duplication or secondary integration event, as the region

downstream of *mgx* is composed of ORFs encoding portions of what would be a full length *mga*-like gene. The 02161A strain, which possess a frameshift mutation in the *simA* gene, also possesses a 117 bp deletion which puts the second *mga*-like ORFs into frame to create a second *mga*-like gene (64). Further support for Mgx regulation in *S. iniae* lies in the presence of a highly conserved 51 bp region upstream of the *simA* gene (5, 64), similar to the conserved Mga binding motif found upstream of GAS M family and C5a peptidase genes (73). Predicted structural analysis of Mgx shows conservation of helix-turn-helix motifs found in Mga (5). While a significant amount of circumstantial evidence exists for Mgx regulation in *S. iniae*, such regulation has yet to be demonstrated.

Therapeutic application of virulence factor discoveries

Review of therapeutic strategies

There are currently four primary therapeutic strategies for combating *S. iniae* infection: antibiotics, probiotics, immunostimulants, and vaccination with killed bacterins. Antibiotic treatment options for *S. iniae* are severely limited in the US and currently there are no FDA approved antibiotics designated for use against *S. iniae*. Work has been done to demonstrate the efficacy of a number of common antibiotics against *S. iniae* infections in fish, including erythromycin (23, 53), enrofloxacin (104), florfenicol (24), oxytetracycline (20, 27), and amoxicillin (25, 26). The therapeutic efficacy of antibiotics to treat *S. iniae* infections appears to be a lesser hurdle compared to achieving regulatory approvals for use, and the tendency of infected fish to not consume medicated feed. Currently only oxytetracycline has been used to treat *S. iniae* outbreaks

in US aquaculture operations, and is done so only as a last resort option under an "extra label" exemption. Others have explored the potential of probiotics to prevent *S. iniae* outbreaks through niche and nutrient competition (2, 15, 16, 42). Dietary supplements including β-glucans (19, 95, 109), distillers dried grains and solubles (63), vitamins C and E (96), and selenium (48) have all been investigated as immunostimulatory additives to increase resistance to *S. iniae*.

The most promising therapeutic strategy for combating *S. iniae* infection lies in vaccination. The quest to develop vaccines to prevent *S. iniae* infections has been met with limited success however (1). One significant hurdle has been the re-emergence of serotypically distinct strains in fish previously vaccinated (4). Other studies suggest that some of the conditions which predispose fish to *S. iniae* infection, such as poor water quality (i.e. high ammonia concentrations) may also act to curtail the adaptive immune response (particularly cellular components) of vaccinated fish (47). In the US, vaccination efforts in aquaculture are limited to autogenous killed bacterin forms of isolates collected from the site of their use as vaccines. Whole cell, formalin-killed bacterin preparations have been the antigen of choice for the majority of *S. iniae* vaccination efforts to date. The use of extracellular products (ECPs) has been shown to increase the efficacy of killed bacterin vaccines (55, 99), though their efficacy when delivered alone has yielded mixed results (32, 99). More comprehensive protection against both *S. iniae* serotypes can be achieved through joint vaccination with representatives of type I and II (55). There are no commercial *S. iniae* vaccines approved for use in the US, but two killed bacterin vaccines, Norvax© Strep *Si* and AquaVac^{TM}

Garvetil ™ (Intervet/Schering Plough Animal Health), are approved for tilapia and are used in Asia.

Live attenuated vaccines

With the increase in knowledge of *S. iniae* virulence mechanisms and a growing number of live attenuated virulence factor mutant strains available, investigation has begun on this alternative and potentially superior vaccination strategy. Live vaccines for other bacterial fish pathogens have demonstrated the advantage of prolonged, unaltered multi-epitope presentation, capable of stimulating a robust humoral and cell-based response (70, 71, 107). Whereas studies using killed bacterin vaccines have demonstrated the importance of ECPs in vaccine preparations (55), live vaccines inherently include these key immunogenic components which are produced *in vivo* in their native state. Another consideration with development of a live vaccine lies in the selection of the virulence factor that is removed. Ideally the virulence determinant that is removed is one integral to pathogenicity, but is not itself an immunodominant epitope whose recognition by the host immune system is critical for generation of protective immunity.

The use of transposon mutagenesis has been a key tool in the search for live vaccine candidates and has led to the discovery of a severely attenuated and highly protective Δ*pgmA* mutant (18). More recent advances in understanding the molecular genetic basis for *S. iniae* virulence has enabled informed mutagenesis of genes which are likely to contribute to *S. iniae* pathogenesis. This approach led to the generation of the highly effective Δ*simA* SiM protein mutant strain, which was capable of providing

complete protection, when delivered IP, against WT *S. iniae* challenge in HSB (64, 67). Another potential advantage of live *S. iniae* vaccines lies in their ability to mimic natural exposure routes in immersion vaccination. Through this more economically tractable delivery method, the Δ*simA* mutant again provided complete immune protection against IP challenge with a lethal dose of WT *S. iniae*, whereas killed bacterin and even effective IP live mutants Δ*pgmA* and Δ*cpsD* showed much diminished protective capacities (67).

Complete attenuation of a live vaccine mutant is a critical property. The Δ*simA* mutant is not without its drawbacks as this strain does generate low levels of mortality (8-16%) through IP or immersion delivery (64, 67) and will require further attenuation before it can be considered a viable commercial vaccine candidate. In addition to safety and efficacy concerns, any commercial live *S. iniae* vaccine will also need to be devoid of any antibiotic resistance markers and be incapable of reversion back to a WT genotype.

Conclusions and future directions

For much of the three decades since *S. iniae* was first isolated, very little was known about the specific virulence mechanisms that this aquatic pathogen possesses. Two of the earliest phenotypic properties, presence of extracellular capsule and hemolysis on blood agar, have now both been characterized as key virulence factors on a molecular genetic level due to sequencing of transposon mutants and genetic information inferred from the earlier characterization of these two virulence determinants in human streptococcal pathogens. With the advent of economical high-throughput sequencing technologies like pyrosequencing and traditional whole genome sequencing efforts,

multiple *S. iniae* virulence determinants have been established in a very short amount of time. We now have identified putative virulence factors involved in every major stage of *S. iniae* pathogenesis and can use this knowledge to help inform the development of novel therapeutic strategies.

One of the most promising applications of research on *S. iniae* virulence and the corresponding generation of virulence factor-deficient mutants lies in the development of live attenuated vaccines. Live vaccines have demonstrated high potential in providing excellent adaptive immune protection and have also shown the ability to serve as superior immersion-based vaccines compared to traditional killed bacterins. Additional applications of understanding virulence could take the form of subunit vaccines based on immunogenic virulence factors, DNA vaccines expressing immunogenic virulence factors, and small molecules or peptides with activity against key virulence determinants.

With the anticipated release of the first *S. iniae* whole genome sequence, our understanding of *S. iniae* virulence and its relationship to other streptococcal pathogens will rapidly accelerate. Future whole genome projects will allow valuable inter-strain genetic comparisons, and will shed light on the genetic basis for serotypic diversity and variation in strain virulence characteristics. The ever increasing demand for seafood, the continued decline in natural fisheries, and the increase in aquaculture operations worldwide suggests that opportunistic bacterial pathogens like *S. iniae* will become more prevalent with time, however, through our increased understanding of *S. iniae* virulence mechanisms we now have the opportunity to develop effective therapeutic strategies to combat *S. iniae* infection.

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Chapter II

A pore-forming cytolysin: Streptolysin S

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Streptococcus iniae β-hemolysin streptolysin S is a virulence factor in fish infection

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ABSTRACT: *Streptococcus iniae* is a leading pathogen of intensive aquaculture operations worldwide, although understanding of virulence mechanisms of this pathogen in fish is lacking. *S. iniae* possesses a homolog of streptolysin S (SLS), a secreted, pore-forming cytotoxin that is a proven virulence factor in the human pathogen *S. pyogenes*. Here we used allelic exchange mutagenesis of the structural gene for the *S. iniae* SLS precursor (*sagA*) to examine the role of SLS in *S. iniae* pathogenicity using *in vitro* and *in vivo* models. The isogenic Δ*sagA* mutant was less cytotoxic to fish blood cells and cultured epithelial cells, but comparable to wild-type (WT) *S. iniae* in adherence/invasion of epithelial cell monolayers and resisting phagocytic killing by fish whole blood or macrophages. In a hybrid striped bass infection model, loss of SLS production led to marked virulence attenuation, as injection of the Δ*sagA* mutant at 1000× the WT lethal dose (LD_{80}) produced only 10% mortality. The neutralization of SLS could represent a novel strategy for control of *S. iniae* infection in aquaculture.

KEY WORDS: *Streptococcus iniae* · Streptolysin S · β-Hemolysin · Virulence factor · Hybrid striped bass · Aquaculture

INTRODUCTION

Streptococcus iniae infection is a threat to the productivity of intensive finfish aquaculture operations worldwide, with annual economic losses measured in the hundreds of millions of dollars. Originally isolated from the freshwater dolphin *Inia geoffrensis* (Pier & Madin 1976), *S. iniae* infects a wide range of fish species such as tilapia (Press et al. 1998), yellowtail (Kitao 1982), trout (Eldar & Ghittino 1999), and hybrid striped bass (Evans et al. 2000). Clinical symptoms of *S. iniae* infection in fish include loss of orientation, lethargy, anorexia, ulcers, exophthalmia, and erratic swimming (Bercovier et al. 1997). Mortality is often attributed to a severe meningoencephalitis (Bercovier et al. 1997). In rare cases, *S. iniae* causes infection in humans who have handled diseased fish (Weinstein et al. 1997). Despite the need for novel approaches to treatments and prevention, phosphoglucomutase (Buchanan et al. 2005) and capsular polysaccharide (Miller & Neely 2005, Locke et al. 2007) are the only 2 *S. iniae* virulence factors characterized to date in the context of fish pathogenesis.

A characteristic helpful in identifying *Streptococcus iniae* in the clinical laboratory is β-hemolysis, a zone of clearing surrounding colonies grown on blood agar media. With this phenotype, *S. iniae* resembles the leading human pathogen *S. pyogenes*, the agent of 'strep throat' and a wide variety of other mucosal or deep tissue infections (Carapetis et al. 2005). The primary factor responsible for β-hemolysis in *S. pyogenes* is streptolysin S (SLS), a small, pore-forming cytotoxin

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Dis Aquat Org 76: 17–26, 2007

with a broad range of membrane targets (Alouf & Loridan 1988, Nizet 2002, Wessels 2005). In addition to SLS, the unrelated, oxygen-sensitive *S. pyogenes* hemolysin, streptolysin O, plays a role in β-hemolysis under anaerobic conditions (Alouf 1980). SLS biosynthesis in *S. pyogenes* is achieved by the products of the 9-gene *sag* (streptolysin-associated genes) operon (*sagA-I*), with sequence characteristics that place the molecule in the family of bacteriocin-like small peptide toxins (Nizet et al. 2000). The 53 amino acid *sagA* gene encodes the putative SLS prepropeptide precursor, while the downstream genes have proposed roles in toxin processing, export, or immunity (Nizet et al. 2000). Recently, precise, in-frame allelic replacement mutagenesis of the structural gene *sagA* encoding the SLS prepropeptide was used to definitively establish the role of SLS production in the pathogenesis of invasive *S. pyogenes* disease using a mouse skin infection model (Datta et al. 2005), thereby corroborating earlier observations using non-hemolytic *S. pyogenes* variants identified through transposon mutagenesis (Betschel et al. 1998).

Recently, genes responsible for the β-hemolytic phenotype of *Streptococcus iniae* have been mapped to a genetic locus closely resembling the 9-gene *sag* operon of *S. pyogenes*, leading to the conclusion that *S. iniae* produces an SLS homolog (Fuller et al. 2002). The candidate *S. iniae* SagA prepropeptide is 54 amino acids in length, and overall sequence similarity across all predicted protein products (SagA-I) of the 2 operons is 73% (Fuller et al. 2002). In this study, we examined the contribution of SLS production to *S. iniae* virulence in fish, coupling precise, in-frame allelic replacement of the *sagA* gene and direct comparisons of the wild-type (WT) *S. iniae* parent strain and isogenic SLS-deficient mutant using *in vitro* and *in vivo* models of disease pathogenesis.

MATERIALS AND METHODS

Bacteria strains, culture, transformation, and DNA techniques. *Streptococcus iniae* strain K288 was isolated from the brain of a diseased hybrid striped bass *Morone chrysops* × *M. saxatilis* (HSB) at the Kent SeaTech aquaculture facility in Mecca, California (Buchanan et al. 2005). Unless otherwise stated, all *S. iniae* (*S. pyogenes*) strains were grown at 30°C (37°C) in Todd-Hewitt broth (THB, Hardy Diagnostics) or agar (THA). Enumeration of colony-forming units (CFU) for *in vitro* assays and *in vivo* infections was performed by serially diluting bacteria in phosphate-buffered saline (PBS) and plating on THA. β-hemolytic activity was assessed on sheep blood agar (SBA) plates (tryptic soy agar with 5% sheep red blood cells). For all assays, overnight cultures of *S. iniae* were diluted 1:10 in fresh THB and grown to mid-log phase (optical density, OD_{600} = 0.40). *S. iniae* strains were rendered electrocompetent for transformation through growth in THB media containing 0.6% glycine following procedures described for *S. agalactiae* (GBS) (Framson et al. 1997); transformants were propagated at 30°C in THB with 0.25 M sucrose. Antibiotic selection was achieved with chloramphenicol (Cm) at 4 µg ml^{-1} or erythromycin (Erm) at 5 µg ml^{-1}. *Escherichia coli* used in cloning were grown at 37°C (unless otherwise stated) while being shaken under aerobic conditions in Luria-Bertani broth (LB, Hardy Diagnostics) or statically on agar (LA). When necessary, *E. coli* were grown in antibiotics: ampicillin (Amp) at 100 µg ml^{-1}, spectinomycin (Spec) at 100 µg ml^{-1}, Erm at 500 µg ml^{-1}, or Cm at 20 µg ml^{-1}. Mach 1 chemically competent *E. coli* (Invitrogen) and electrocompetent MC1061 *E. coli* used in transformations were recovered through growth at 30°C in SOC media (Invitrogen). A PureLink Quick Plasmid Miniprep Kit (Invitrogen) was used to isolate plasmids propagated in *E. coli*. *S. iniae* genomic DNA was isolated using the UltraClean DNA Isolation Kit (MoBio).

Cell lines and culture conditions. The adherent carp leukocyte culture (CLC) carp monocytic/macrophage cell line (European Collection of Cell Cultures no. 95070628) and the WBE27 white bass embryonic epithelial cell line (American Type Culture Collection no. CRL-2773; Shimizu et al. 2003) were grown at 28°C with 5% CO_2. Cells were maintained in 125 ml tissue culture flasks in Dulbecco's modified Eagle's medium (DMEM, Gibco) containing 10% heat-inactivated fetal bovine serum (FBS, Gibco).

Allelic exchange mutagenesis. Allelic exchange mutagenesis (see Fig. 1) of *Streptococcus iniae* strain K288 was carried out as previously described (Buchanan et al. 2006) with the only significant modification being the incorporation of the Gateway cloning system (Invitrogen). PCR was used to amplify ~400 bp of *S. iniae* chromosomal DNA fragments directly upstream and downstream of *sagA* (GenBank accession no. AF465842), with primers adjacent to *sagA* constructed to possess 25 bp 5′-extensions corresponding to the 5′- and 3′- ends of the chloramphenicol acetyltransferase (*cat*) gene from pACYC (Nakano et al. 1995), respectively. The upstream (Up) and downstream (Down) PCR products were then combined with a 660 bp amplicon of the complete *cat* gene using fusion PCR (Wang et al. 2002). The resultant PCR amplicon containing an in-frame substitution of *sagA* with *cat* was subcloned into the Gateway entry vector pCR 8/GW/TOPO (Invitrogen) and transformed into Mach1 *Escherichia coli* (Invitrogen). Plasmid DNA was extracted and a Gateway LR recombination reaction

performed to transfer the fusion PCR amplicon into the corresponding Gateway entry site of a temperature-sensitive knockout vector pKODestErm (a derivative of pHY304 [Chaffin et al. 2000] created for Gateway cloning), thereby generating the knockout plasmid pKOsagA. The pKOsagA construct was introduced into WT *S. iniae* by electroporation; transformants were identified at 30°C by Erm selection and then shifted to the nonpermissive temperature for plasmid replication (37°C). Differential antibiotic selection (Cm^R and Erm^S) was used to identify candidate allelic exchange mutants. Targeted in-frame replacement of *sagA* was confirmed unambiguously by PCR reactions documenting the desired insertion of *cat* and absence of *sagA* sequence in chromosomal DNA isolated from the final isogenic mutant, K288Δ*sagA*.

Complementation studies. PCR was used to amplify the *sagA* gene and ~200 bp of upstream sequence containing its predicted promoter and ribosomal binding site from *Streptococcus iniae* strain K288. The amplicon was cloned into pCR8/GW/TOPO as described above. A Gateway LR recombination reaction was used to transfer the PCR insert from the entry vector into the Gram-positive expression plasmid, pDCerm (Jeng et al. 2003), modified to contain Gateway recombination sites (pDESTerm), thus creating complementation vector psagA. The plasmid was successfully transformed into the Δ*sagA S. iniae* and Δ*sagA S. pyogenes* (Datta et al. 2005) mutant strains as confirmed by PCR mapping and antibiotic resistance profiles.

Growth rate. Mid-log phase cultures of WT *Streptococcus iniae* and the isogenic Δ*sagA* mutant were diluted 1:10 in a 96-well plate. Growth was monitored spectrophotometrically at 600 nm every 30 min for 5.5 h.

Invasion and adherence assays. Invasion and adherence assays were performed in collagenized 96-well tissue culture plates (Costar) using confluent monolayers of WBE27 white bass epithelial cells. Bacteria in DMEM containing 2% FBS were added to each well to achieve a multiplicity of infection (MOI) of 10 (bacteria:cells). Following centrifugation at 350 × *g* for 5 min, the plate was incubated for 60 min at 28°C with 5% CO_2. The cells were then washed twice with DMEM containing 2% FBS and incubated in fresh DMEM with 20 µg ml^{-1} penicillin (Invitrogen) and 200 µg ml^{-1} gentamicin (Invitrogen) for 60 min to kill extracellular bacteria. Cells were then washed twice with DMEM containing 2% FBS and lysed by trituration in 100 µl of 0.01% Triton X-100 (Sigma). Surviving intracellular bacteria were quantified by plating serial dilutions of lysed cell supernatant on THA. Adherence assays were carried out in a similar manner except that no antibiotics were used, and the bacteria were added to the cells for 30 min and washed 5 times with DMEM containing 2% FBS to remove non-adherent bacteria prior to enumeration of CFU. The optimal MOI for all culture-based assays was experimentally determined.

Macrophage killing assay. Monolayers of carp macrophages (CLC) were grown as described for the invasion and adherence assays. Bacteria were diluted in DMEM containing 2% FBS, added to the cells at an MOI of 0.01, and incubated at 28°C for 1, 7, or 20 h. Cells were lysed and plated as described above for invasion and adherence assays. Percent survival was calculated based on the initial inoculum.

Whole blood survival. Blood was extracted via a syringe from the caudal vein of 3 HSB and collected in a heparinized tube. Three hundred µl of each blood sample were immediately added to 2 ml siliconized microcentrifuge tubes with ~300 CFU *Streptococcus iniae* suspended in 100 µl PBS. Tubes were incubated with shaking at 30°C for 1 h. Two 100 µl aliquots from each blood sample were spread onto THA to enumerate surviving bacteria. Survival was calculated as a percentage of remaining bacteria relative to the starting inoculum.

Cytotoxicity assays. Collagenized 96-well tissue culture plates (Costar) were seeded with 1×10^5 WBE27 or CLC cells in 200 µl RPMI (Gibco) with 2% FBS and bacteria added at an MOI of 100. Plates were spun at 350 × *g* for 5 min to ensure bacteria/cell contact. Plates were incubated at 28°C for 5 h before analysis. One µl of a 1:10 000 dilution of SYTOX Orange (Invitrogen) was added to each well. Cells were observed with a Zeiss Axiovert 40 inverted microscope under bright field conditions and through fluorescence microscopy using a standard rhodamine filter set at 400× magnification. This experiment was repeated 3 times with identical results.

Total blood cell hemolysis. Fresh, heparinized, whole HSB blood was washed 3 times in 20 volumes of PBS and resuspended as a 2% solution (v/v). In a 96-well round bottom plate, a mid-log culture of WT *Streptococcus iniae* and the Δ*sagA* mutant were aliquoted in quadruplicate in volumes of 100 µl. Each well then received 100 µl of the 2% fish blood solution. Background lysis was measured in wells containing only blood cells and THB. Complete lysis was measured by wells containing blood cells, plain THB, and 2 µl of Triton X-100. Plates were incubated at 30°C for 2 h and then at 4°C for 2 h. Following centrifugation at 1500 × *g* for 5 min, 100 µl from each well were added to a new flat-bottom 96-well plate, and the optical density was read at 405 nm in a microplate reader (Molecular Devices).

Dose-response challenge. Comparative *in vivo* virulence analysis of WT *Streptococcus iniae* and the Δ*sagA* mutant was performed using an infection chal-

Dis Aquat Org 76: 17–26, 2007

lenge of juvenile (~27 g) HSB. Fish were maintained at 25°C in ~75 l flow-through tanks. Overnight cultures of each strain were diluted 1:10 and grown to mid-log phase. Bacteria were pelleted, resuspended, and diluted in PBS to the desired dose in a 100 µl injection volume. Groups of 20 fish were injected intraperitoneally (IP) with either PBS alone (control), 3×10^5 CFU of WT *S. iniae*, or a series of doses of the Δ*sagA* mutant ranging from 3×10^5 to 3×10^8 CFU. Survival was monitored for 10 d. All fish challenges were carried out in an Association for Assesment and Accreditation of Laboratory Animal Care (ALAAC) certified facility following Institutional Animal Care and Use Committee (IACUC) approved protocols.

Characterization of *in vivo* fish infection. The infectious process of WT *Streptococcus iniae* and the Δ*sagA* mutant was characterized through *in vivo* challenge in HSB. Bacterial suspensions were prepared as described above. Groups of 30 fish were injected IP with 3×10^5 CFU or PBS for controls. At 0.25, 1, 2, 3, 4, 5, and 9 d, 4 fish from each group were sampled for presence of bacteria in the blood, spleen, and brain (no remaining WT-infected fish were available to sample on Day 9). Samples were stored briefly on ice, weighed in microcentrifuge tubes, and resuspended with a 5-fold quantity of PBS (v/w). Spleen and brain tissue were manually homogenized, diluted in PBS, and plated on THA. Blood CFUs were measured by plating dilutions from an initial 1:10 dilution of whole blood in PBS. Bacterial colonies from each CFU plating sample were streaked onto SBA to confirm corresponding hemolytic or non-hemolytic phenotypes. Sagittal brain tissue samples were taken from 3 fish in each treatment group at 3 and 4 d post challenge. Samples were placed in cassettes and preserved in 10% buffered formalin for histological analysis. Cassettes were embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E).

Statistical analyses. Data analyses were performed using the statistical tools included with GraphPad Prism (GraphPad Software). *In vitro* assay data were analyzed using unpaired 2-tailed *t*-tests. Fish infection survival data were analyzed using a Logrank Test. Fish tissue and blood CFU data were log-normalized prior to statistical analyses. A value of $p < 0.05$ was considered statistically significant. *In vitro* assays were repeated 3 times with equivalent results, in quadruplicate, and data presented (mean ± standard error of the mean, SEM) are from a single representative assay.

RESULTS

Allelic replacement and complementation of *Streptococcus iniae sagA*

We were successful in generating an in-frame allelic replacement of *sagA* with the *cat* gene in the *Streptococcus iniae* chromosome (Fig. 1). Colonies of the *S. iniae* Δ*sagA* mutant were non-hemolytic on SBA, confirming the requirement for *sagA* in *S. iniae* SLS production (Fig. 2A). Complementation of the Δ*sagA* mutant with the *S. iniae sagA* gene *in trans* on the plasmid psagA restored the hemolytic phenotype on SBA (Fig. 2A), excluding polar effects on downstream genes in the SLS biosynthetic operon. Heterologous expression of the *S. iniae sagA* gene also restored hemolytic function to an *S. pyogenes* Δ*sagA* allelic exchange mutant (Datta et al. 2005), demonstrating the functional homology of the SagA toxins from the fish and human pathogens. The *sagA* deletion mutation did not negatively impact the *S. iniae* growth profile (Fig. 2B).

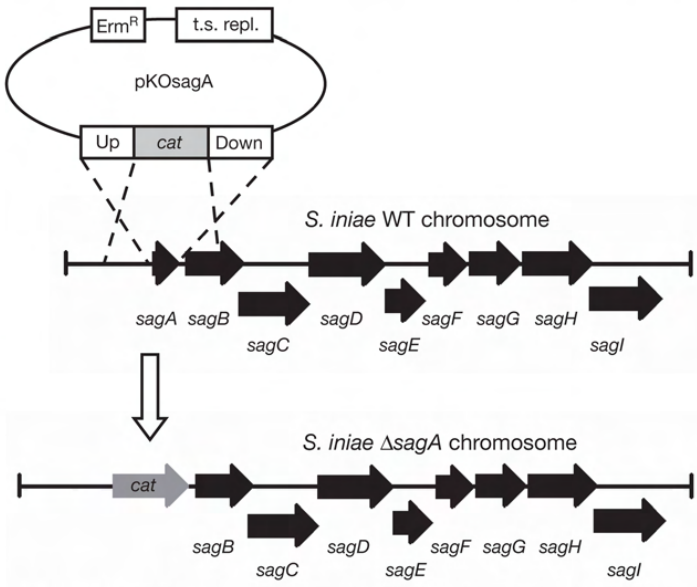

Fig. 1. Allelic exchange mutagenesis scheme for replacement of the *sagA* gene of the steptolysin S (SLS) operon with *cat*. The pKOsagA plasmid contains the fusion PCR composed of ~400 bp *sagA*-flanking regions upstream (Up) and downstream (Down) nesting the *cat* gene in between. The plasmid also confers erythromycin resistance (Erm^R) and a temperature-sensitive origin of replication (t.s. repl.). Through 2 independent single crossover events, the *Streptococcus iniae sagA* gene is precisely replaced in-frame by the *cat* gene. WT: wild-type

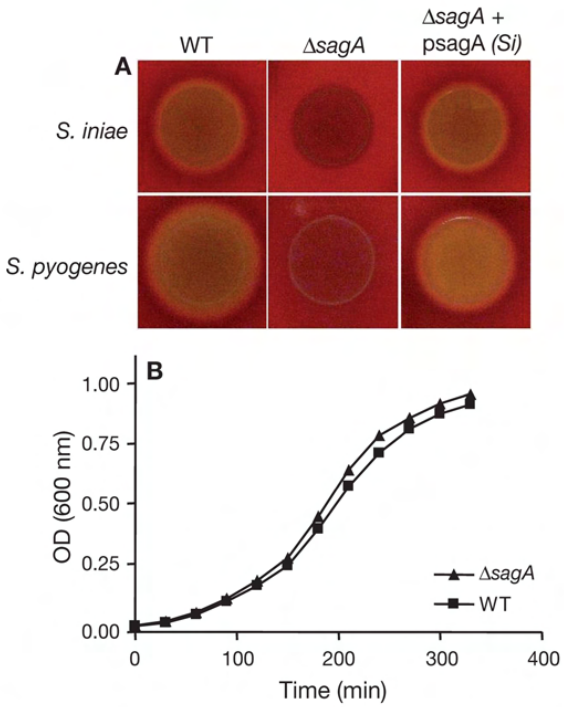

Fig. 2. Functional validation of the Δ*sagA* mutant. (A) Deletion of *Streptococcus iniae sagA* (Δ*sagA*) results in loss of hemolytic activity. The SLS⁻ mutant phenotype, in both *S. iniae* and *S. pyogenes*, can be rescued by complementation using *S. iniae sagA* on a plasmid, psagA. (B) Growth rate comparison between the wild-type (WT) and the isogenic Δ*sagA* mutant strain in Todd-Hewitt broth (THB). OD: optical density

SLS does not contribute to *Streptococcus iniae* epithelial cell invasion or adherence

The ability of streptococcal pathogens to adhere to and invade epithelial cell barriers is felt to play an important role in the development of invasive infection (Molinari & Chatwall 1999). For example, host cellular invasion is promoted by the activities of the β-hemolysin of *Streptococcus agalactiae* (Doran et al. 2002) and pneumolysin of *S. pneumoniae* (Cockeran et al. 2002); however, SLS did not serve a similar function in *S. pyogenes* (Datta et al. 2005). We used the epithelial white bass cell line WBE27 to compare the cellular adherence and invasion of WT *S. iniae* and the isogenic Δ*sagA* mutant, and found no significant differences (p = 0.6347, 0.1323; Fig. 3A). Thus, SLS expression by *S. iniae* does not appear to itself promote host epithelial cells interactions in this *in vitro* model system.

SLS does not promote *Streptococcus iniae* phagocyte resistance

SLS of *Streptococcus pyogenes* contributes to bacterial survival in freshly isolated human whole blood, presumably through interference with the function of circulating phagocytic cells such as neutrophils (Datta et al. 2005). However, upon comparison of WT and Δ*sagA S. iniae* survival in freshly isolated blood from

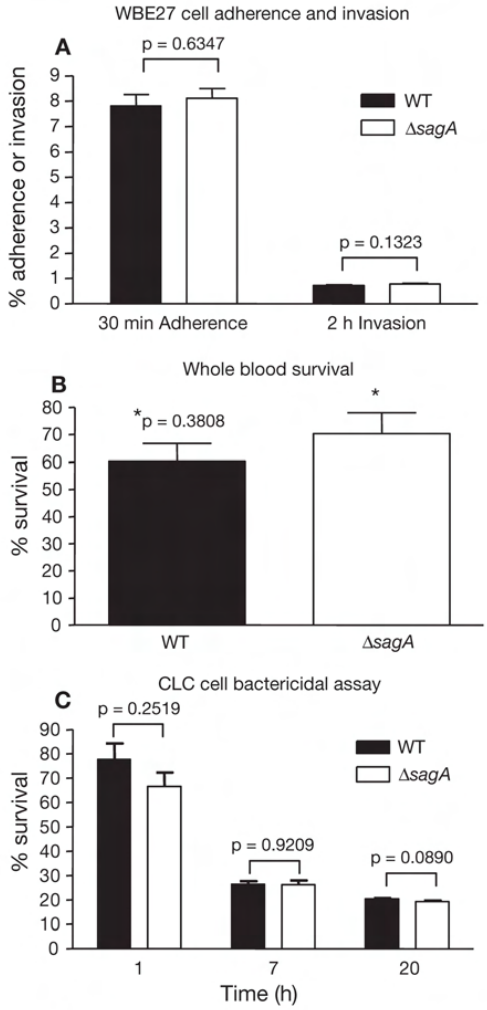

Fig. 3. Lack of SLS contribution to certain *Streptococcus iniae* virulence phenotypes *in vitro*. (A) Adherence and invasion characteristics of wild-type (WT) *S. iniae* and the Δ*sagA* (SLS⁻) mutant for fish epithelial cell line WBE27. (B) Survival of WT and the Δ*sagA* mutant in whole hybrid striped bass (HSB) blood (1 h), or (C) upon co-incubation with carp leukocyte culture (CLC) fish macrophage/monocytes for 1, 7, or 20 h

Dis Aquat Org 76: 17–26, 2007

HSB, no significant differences were observed within 1 h ($p = 0.3808$; Fig. 3B). The *S. agalactiae* β-hemolysin promotes bacterial survival in murine macrophages (Liu et al. 2004). Yet, assessment of WT and Δ*sagA S. iniae* survival in a fish CLC monocyte/macrophage killing assay again revealed no differences through a series of different incubation time points (Fig. 3C). Thus, SLS expression by *S. iniae* is likely not a major contributor to the pathogen's ability to resist phagocyte-mediated clearance.

SLS production promotes *Streptococcus iniae* fish cell cytotoxicity

SLS of *Streptococcus pyogenes* is one of the most potent known cytotoxins, with broad spectrum activity against a wide variety of mammalian cell membranes (Alouf & Loridan 1988, Nizet 2002, Wessels 2005). To assess the cytolytic potential of *S. iniae* SLS in the context of fish infection, we incubated the WT and Δ*sagA* mutant with cultured white bass epithelial cells (WBE27) or carp macrophages (CLC) for 5 h and then measured cell viability by a fluorescent assay. In both cases, a striking decrease in the number of non-viable cells was observed in wells treated with the SLS-deficient *S. iniae* mutant (Fig. 4A). Extending these studies, the hemolytic activity of *S. iniae* was assessed in freshly isolated HSB blood. The *S. iniae* Δ*sagA* mutant showed little hemolytic activity above background whereas the WT parent strain was capable of lysing a majority of the cells (Fig. 4B). These results indicate that SLS is a major contributor to the cytotoxic activity of *S. iniae* against a variety of fish-derived cell types.

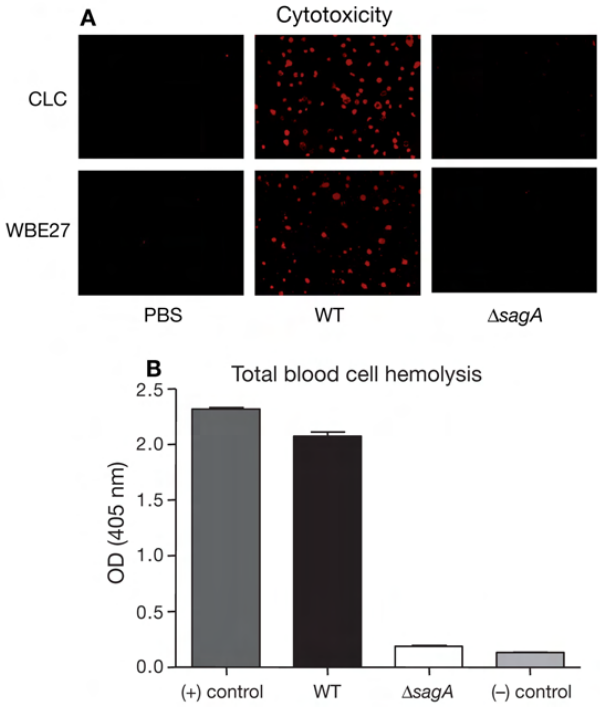

Fig. 4. Assessment of cytotoxic effects of SLS on fish epithelial, macrophage, and blood cells. (A) Cytotoxicity of wild-type (WT) *Streptococcus iniae* and the Δ*sagA* mutant toward white bass epithelial (WBE27) and carp leukocyte culture (CLC) cells following a 5 h incubation at a multiplicity of infection (MOI) of 100 using SYTOX Orange to stain for DNA in dead cells (red). PBS: phosphate-buffered saline. (B) Hemolytic activity; the optical density (OD) of total hybrid striped bass (HSB) blood cell supernatant was measured after incubation with plain THB containing Triton X-100 (positive control), WT *S. iniae*, the *S. iniae* SLS$^-$ mutant (Δ*sagA*), or with plain THB (negative control)

SLS expression contributes to *Streptococcus iniae* virulence in fish

An HSB challenge model was used to test the requirement of SLS expression in *Streptococcus iniae* pathogenicity. Intraperitoneal injection of 3×10^5 CFU produced 80% fish mortality by Day 6 (Fig. 5). In contrast, the Δ*sagA* mutant *S. iniae* was markedly attenuated for virulence, producing no deaths at the equivalent challenge dose nor a 10-fold higher dose, and only 5 or 10% mortality at challenge doses 100-fold and 1000-fold higher, respectively (Fig. 5). To further characterize the aborted infectious process associated with loss of SLS, bacterial counts were measured in

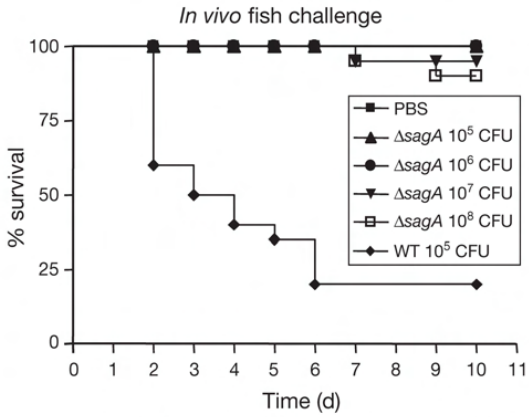

Fig. 5. Kaplan-Meier survival plot of hybrid striped bass (HSB) infected with wild-type (WT) *Streptococcus iniae* (3×10^5 CFU), the Δ*sagA* (SLS$^-$) mutant (3×10^5, 10^6, 10^7, or 10^8 CFU), or phosphate-buffered saline (PBS)

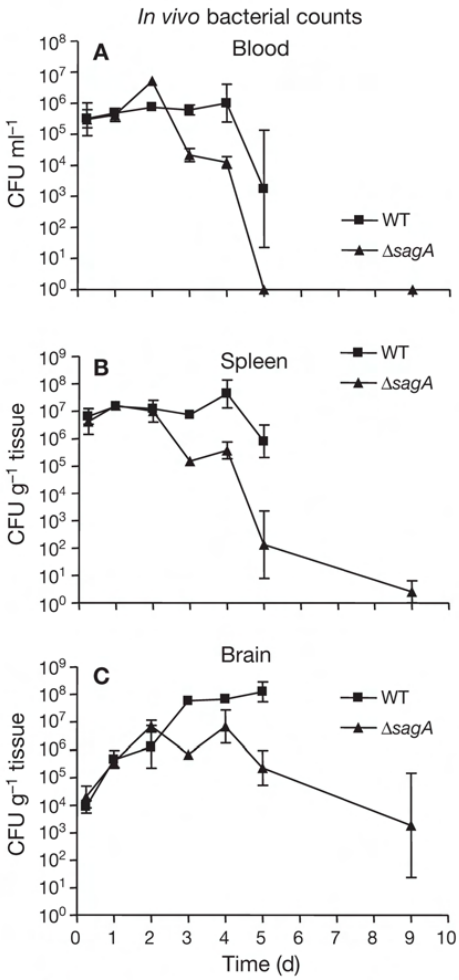

Fig. 6. Characterization of the systemic dissemination of wild-type (WT) *Streptococcus iniae* and the isogenic Δ*sagA* mutant in hybrid striped bass (HSB). CFUs are averages of levels detected in the (A) blood, (B) spleen, and (C) brain of 4 fish per group at 0.25, 1, 2, 3, 4, 5, and 9 d post challenge

the blood, spleen, and brain of HSB at various time points after challenge with the WT strain or Δ*sagA* mutant. All recovered bacteria were plated on SBA to confirm their identity as *S. iniae* with the expected presence or absence of SLS expression. Whereas CFU counts for both strains were similar in all 3 HSB tissues for the first 48 h after challenge, levels of the Δ*sagA* mutant began to decrease in subsequent days whereas those of the WT remained at high levels (Fig. 6). Curiously, while the Δ*sagA* mutant was cleared completely from the blood by Day 5 and from the spleen by Day 9 after infection, 2 out of 4 HSB

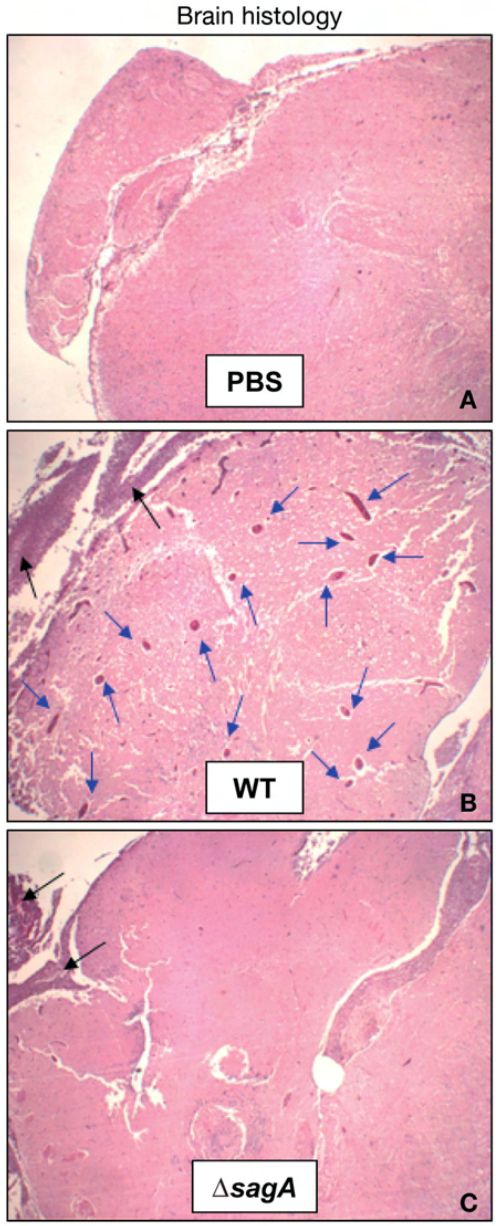

Fig. 7. Histological analysis of brain tissue of hybrid striped bass (HSB). Sagittal brain sections were collected 4 d post challenge from fish injected with (A) phosphate-buffered saline (PBS), (B) 3×10^5 wild-type (WT) *Streptococcus iniae* or (C) the Δ*sagA* mutant. Sample sections were stained with hematoxylin and eosin and visualized at 40× magnification. Representative images of cerebral tissue are shown for each treatment group. (B,C) WT and *sagA*-infected fish show meningeal inflammation (black arrows). (B) In addition, WT-infected fish show significant dilation and congestion of red blood cells in the cerebral blood vessels (blue arrows)

Dis Aquat Org 76: 17–26, 2007

infected with the Δ*sagA* mutant still harbored high levels of bacteria in the brain 9 d after infection (>10^6 CFU g^{-1}), yet exhibited no mortality nor clinical signs of meningoencephalitis (i.e. lethargy, exophthalmia, loss of orientation) throughout the 10 d challenge period. Histological analysis of brain sections from WT- and Δ*sagA* mutant-infected fish on Days 3 and 4 post challenge showed comparable levels of a mixed inflammatory cell infiltrate on the meningeal surface; however, dilation of cerebral vessels with foci of thrombosis were present in the WT-challenged animals but absent in those infected with the SLS-deficient mutant (Fig. 7).

DISCUSSION

Molecular genetic methods have only recently been applied to explore the potential virulence mechanisms by which the leading aquaculture pathogen *Streptococcus iniae* produces systemic infection in aquacultured fish. Similar to other important streptococcal pathogens of humans (e.g. *S. pyogenes*, *S. agalactiae*) or animals (e.g. *S. suis*, *S. equi*), *S. iniae* exhibits a β-hemolytic phenotype on blood agar, indicating the elaboration of one or more membrane-disrupting toxins. Recently, the *S. iniae* β-hemolysin was identified as a homolog of the *S. pyogenes* toxin SLS (Fuller et al. 2002). Here we applied allelic replacement mutagenesis of the toxin-encoding gene *sagA* to establish that SLS expression plays a key role in the pathogenesis of *S. iniae* fish infection.

The loss of SLS production produced a profound overall attenuation in the virulence potential of *Streptococcus iniae* in the HSB infection model (only 10% mortality at a challenge dose 1000-fold higher than the 80% lethal dose for the WT strain). Based on the existing literature regarding the action of streptococcal pore-forming toxins during infection, we explored 3 general categories by which the *S. iniae* SLS toxin could harm the fish: promoting cellular adherence and invasion, increasing resistance to phagocytic killing, and/or producing direct cytolytic injury to cell and tissues. Direct comparison of WT and Δ*sagA* mutant *S. iniae* using our *in vitro* fish cell model systems suggest that the latter activity may be the most significant, as SLS expression contributes directly to the cell death of fresh blood cells as well as cultured fish macrophages and epithelial cells.

In producing systemic infection of multiple organs including the central nervous system (CNS), pathogenic streptococci reveal a capacity to penetrate epithelial and/or endothelial barriers (Molinari & Chhatwal 1999) and to resist rapid phagocytic clearance (Voyich et al. 2004). We found that *Streptococcus iniae* was able to efficiently adhere to and invade monolayers of the cultured fish epithelial cell line WBE27, but that SLS expression was not required for this phenotype. Similarly, the ability of *S. iniae* to survive killing in fresh HSB blood or by CLC macrophages was not influenced by SLS expression. This is in contrast to findings for SLS-deficient *S. pyogenes* survival in whole human blood (Datta et al. 2005). These *in vitro* observations appeared to correlate with findings in the early stages (first 48 h) of infection *in vivo*, as the *S. iniae* Δ*sagA* mutant established similar levels of bacteremia and penetrated the blood-brain barrier to access brain tissues to an equivalent degree as the parent strain.

Streptococcus iniae disease in HSB and tilapia represents a systemic septicemia with bacterial cocci evident in the plasma, circulating phagocytes, and most organs including the spleen, kidneys, and prominently, the CNS (Evans et al. 2000, McNulty et al. 2003). Acute mortality associated with WT infection was eliminated by deletion of SLS, although significant numbers of the Δ*sag*A mutant persisted in the brain for several days. Brain sections from both WT and the Δ*sagA* mutant showed a mixed inflammatory infiltrate on the meningeal surface. However, dilation of brain vessels with thrombus formation seen with WT infection was absent in the fish infected with the mutant strain, suggesting that SLS expression may promote cerebrovascular endothelial injury or dysfunction. The pore-forming β-hemolysins of *S. agalactiae* and *S. suis*, CNS pathogens of humans and pigs, respectively, both contribute to injury of cerebrovascular endothelial cells (Nizet et al. 1997, Vanier et al. 2004).

In sum, we found that the β-hemolysin SLS, used commonly as a phenotypic tool in the clinical microbiologic diagnosis of *Streptococcus iniae* infection, is also a critical factor in disease pathogenesis. Direct cytotoxicity against fish cells is likely a major factor underlying the virulence role of the *S. iniae* SLS toxin. However, the RNA encoding the SagA prepropeptide for the SLS toxin in *S. pyogenes* has further been implicated in the pre- and post-translational regulation of other virulence factors of the human pathogen (Li et al. 1999, Mangold et al. 2004). In the future, when additional genetically encoded virulence phenotypes are demonstrated for *S. iniae*, our isogenic allelic replacement mutant may be useful for parallel explorations of global gene regulation functions. As *S. iniae* infection remains a significant threat to the economic viability of intensive aquaculture operations worldwide, a more comprehensive understanding of the specific virulence factors required for *S. iniae* pathogenesis, including SLS, can point to new targets for anti-infective therapy or vaccine development.

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Chapter III

An antiphagocytic cloak: Capsular polysaccharide

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Streptococcus iniae Capsule Impairs Phagocytic Clearance and Contributes to Virulence in Fish▽

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Surface capsular polysaccharides play a critical role in protecting several pathogenic microbes against innate host defenses during infection. Little is known about virulence mechanisms of the fish pathogen *Streptococcus iniae*, though indirect evidence suggests that capsule could represent an important factor. The putative *S. iniae* capsule operon contains a homologue of the *cpsD* gene, which is required for capsule polymerization and export in group B *Streptococcus* and *Streptococcus pneumoniae*. To elucidate the role of capsule in the *S. iniae* infectious process, we deleted *cpsD* from the genomes of two virulent *S. iniae* strains by allelic exchange mutagenesis to generate the isogenic capsule-deficient Δ*cpsD* strains. Compared to wild-type *S. iniae*, the Δ*cpsD* mutants had a predicted reduction in buoyancy and cell surface negative charge. Transmission electron microscopy confirmed a decrease in the abundance of extracellular capsular polysaccharide. Gas-liquid chromatography–mass spectrometry analysis of the *S. iniae* extracellular polysaccharides showed the presence of L-fucose, D-mannose, D-galactose, D-glucose, D-glucuronic acid, *N*-acetyl-D-galactosamine, and *N*-acetyl-D-glucosamine, and all except mannose were reduced in concentration in the isogenic mutant. The Δ*cpsD* mutants were highly attenuated in vivo in a hybrid striped bass infection challenge despite being more adherent and invasive to fish epithelial cells and more resistant to cationic antimicrobial peptides than wild-type *S. iniae*. Increased susceptibility of the *S. iniae* Δ*cpsD* mutants to phagocytic killing in whole fish blood and by a fish macrophage cell line confirmed the role of capsule in virulence and highlighted its antiphagocytic function. In summary, we report a genetically defined study on the role of capsule in *S. iniae* virulence and provide preliminary analysis of *S. iniae* capsular polysaccharide sugar components.

Streptococcus iniae was first isolated from an Amazon River dolphin (*Inia geoffrensis*) in the 1970s (38). Though *S. iniae* infections in humans can occur in the form of cellulitis resulting from a fish handling injury (52), this bacterium is primarily problematic as an aquatic pathogen. Over 30 freshwater and saltwater fish species have demonstrated susceptibility to the disease, including such economically important species as tilapia (39), yellowtail (26), trout (16), and hybrid striped bass (HSB) (17). Common clinical symptoms of *S. iniae* infection in fish include loss of orientation, lethargy, ulcers, exophthalmia, and erratic swimming (6). Mortality resulting from *S. iniae* infection is often attributed to meningoencephalitis and is responsible for aquaculture losses measured in the hundreds of millions of dollars annually. *S. iniae* can also cause significant disease outbreaks in wild fish populations (53). The virulence mechanisms of *S. iniae* are largely unknown.

Our preliminary screening of an *S. iniae* transposon mutant library in HSB indicated that genes involved in capsule synthesis may be associated with virulence. Among other genes, we found that the disruption of the phosphoglucomutase gene resulted in a putative alteration of cell wall architecture, capsule expression, and virulence attenuation associated with increased susceptibility to antimicrobial peptides (AMPs) compared to wild-type (WT) *S. iniae* in an HSB infection challenge (8). A similar *S. iniae* transposon library screen in zebrafish revealed that mutations leading to decreased buoyancy (reflective of potential defects in capsule synthesis) represented a significant proportion of the attenuated mutants (31).

The molecular basis for capsule synthesis has been studied in several streptococcal species (29). For *S. iniae*, electron micrographs show the presence of an extracellular capsule (3, 8), and a putative capsule operon sequence has been identified (GenBank accession no. AY904444). Multiple streptococcal capsule operons contain a conserved group of genes (*cpsA* to *-E*) that are collectively responsible for capsule chain length determination and export (11, 21, 32). The *cpsD* gene, encoding an autophosphorylating protein tyrosine kinase, has been identified as required for capsule synthesis in *Streptococcus pneumoniae* (4, 33). In group B *Streptococcus* (GBS) (*S. agalactiae*), allelic replacement of *cpsD* resulted in a 91% reduction in capsular polysaccharide (11). Blast analysis (1) of the predicted amino acids of the *S. iniae* CpsD homologue (GenBank accession no. AAY17296) showed that it has 63% identity and 82% similarity to GBS CpsD. To gain a better understanding of the potential role of capsule in *S. iniae* infection, we deleted the *S.*

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iniae cpsD homologue and compared the resulting isogenic mutant to the WT parent strain by using biochemical techniques and a series of in vitro and in vivo models of disease pathogenesis.

MATERIALS AND METHODS

Bacterial strains, culture, transformation, and DNA techniques. WT *S. iniae* strain K288 was isolated from the brain of a diseased HSB at the Kent SeaTech aquaculture facility in Mecca, CA (8). *S. iniae* strain 94-426 was originally isolated from a diseased tilapia. All *S. iniae* strains were grown at 30°C (unless otherwise stated) in Todd-Hewitt broth (THB) (Hardy Diagnostics) or Todd-Hewitt agar (THA). Enumeration of CFU for in vitro assays and in vivo infections was performed by plating dilutions on THA. Beta-hemolytic activity of *S. iniae* was assessed on sheep blood agar plates (tryptic soy agar with 5% sheep red blood cells added). In all assays, overnight cultures of *S. iniae* were diluted 1:10 in fresh THB and grown to mid-log phase (optical density at 600 nm of 0.40). *S. iniae* strains were rendered electrocompetent for transformation through growth in THB containing 0.6% glycine according to procedures described for GBS (18); transformants were propagated at 30°C in THB with 0.25 M sucrose. Antibiotic selection was achieved with chloramphenicol (Cm) at 4 μg/ml, erythromycin (Erm) at 5 μg/ml, or spectinomycin at 100 μg/ml. *Escherichia coli* used in cloning was grown at 37°C (unless otherwise stated), with shaking, under aerobic conditions in Luria-Bertani broth (Hardy Diagnostics) or statically on Luria agar. When necessary, *E. coli* was grown in antibiotics, i.e., ampicillin at 100 μg/ml, spectinomycin at 100 μg/ml, Erm at 500 μg/ml, or Cm at 20 μg/ml. Mach1 chemically competent *E. coli* (Invitrogen) and MC1061 electrocompetent *E. coli* used in transformations were recovered through growth at 30°C in SOC medium (Invitrogen). A PureLink Quick plasmid miniprep kit (Invitrogen) was used to isolate plasmids propagated in *E. coli*. *S. iniae* genomic DNA was isolated using the UltraClean DNA isolation kit (MoBio).

Cell lines and culture conditions. The adherent carp monocytic/macrophage cell (CLC) line (European Collection of Cell Cultures 95070628) and the WBE27 white bass embryonic epithelial cell line (ATCC CRL-2773) (48) were grown at 28°C with 5% CO_2. Cells were passaged fewer than 10 times and maintained in 125-ml tissue culture flasks in Dulbecco modified Eagle medium (DMEM)(Gibco) containing 10% heat-inactivated fetal bovine serum (FBS) (Gibco).

Statistical analyses. Data analyses were performed using the statistical tools included with GraphPad Prism (GraphPad Software, Inc.). In vitro assay data were analyzed using unpaired two-tailed *t* tests. Fish infection survival data were analyzed using a log rank test. A P value of <0.05 was considered statistically significant. In vitro assays were repeated three times, in quadruplicate, and the data presented (means ± standard errors of the means [SEM]) are from a single representative assay.

Allelic exchange mutagenesis. Allelic exchange mutagenesis of *S. iniae* strains K288 and 94-426 was carried out as previously described (7), with the only significant modification being the use of the Gateway cloning system (Invitrogen). For PCR, all primers were designed based on the *cpsD* gene region of the *S. iniae* capsule operon deposited under GenBank sequence accession number AY904444. PCR was used to amplify ~400 bp of *S. iniae* chromosomal DNA fragments directly upstream and downstream of *cpsD*, with primers adjacent to *cpsD* constructed to possess 25-bp 5′ extensions corresponding to the 5′ and 3′ ends of the chloramphenicol acetyltransferase (*cat*) gene from pACYC (34), respectively. The upstream and downstream PCR products were then combined with a 660-bp amplicon of the complete *cat* gene by using fusion PCR (51). The resultant PCR amplicon containing an in-frame substitution of *cpsD* with *cat* was subcloned into the Gateway entry vector pCR 8/GW/TOPO and transformed into chemically competent Mach1 *E. coli* (Invitrogen). Plasmid DNA was extracted, and a Gateway LR recombination reaction was performed to transfer the fusion PCR amplicon into the corresponding Gateway entry site of a temperature-sensitive knockout vector, pKODestErm (created for Gateway cloning from pHY304 (9), to generate the knockout plasmid pKOcpsD. Following propagation in MC1061 *E. coli*, the pKOcpsD construct was introduced into WT *S. iniae* by electroporation. Transformants were identified at 30°C by Erm selection and shifted to the nonpermissive temperature for plasmid replication (37°C). Differential antibiotic selection (Cm^r and Erm^s) was used to identify candidate allelic exchange mutants. Targeted in-frame replacement of *cpsD* was confirmed unambiguously by PCRs documenting the desired insertion of *cat* and absence of *cpsD* sequence in chromosomal DNA isolated from both of the final Δ *cpsD* mutants and by phenotype, with the observation of rapid sinking in liquid culture.

Transmission electron microscopy. Capsular polysaccharide of mid-log-phase WT K288 and K288 Δ*cpsD* was visualized via transmission electron microscopy using a lysine acetate fixation protocol as previously described (23). The only notable deviation in this protocol was the use of an overnight room temperature incubation in the second fixation step. Samples were embedded in LR White (Fluka), sectioned, and counterstained with uranyl acetate. Grids were viewed and photographed using a JEOL 1200EX II transmission electron microscope (JEOL, Peabody, MA) at a magnification of ×15,500 and an acceleration voltage of 80 kV.

Cytochrome *c* assay. Anionic cell surface charge was measured through a cytochrome *c* binding assay as previously described (8). An overnight culture of each *S. iniae* strain was diluted 1:10 and grown to mid-log phase. Five milliliters of the bacteria was pelleted at 13,000 × *g* for 5 min and resuspended in 1 ml of MOPS (morpholinepropanesulfonic acid) (pH 7.0). The bacteria were pelleted and then resuspended in 450 μl of MOPS and 50 μl of 10-mg/ml cytochrome *c* (Sigma). The solution was vortexed and incubated at room temperature for 15 min. The bacteria were pelleted, and 200 μl of the supernatant was added to a flat-bottom 96-well plate. The amount of unbound cytochrome *c* was determined by absorbance of the supernatant at 530 nm.

Growth rate analysis and hemolytic activity. Mid-log-phase cultures of WT *S. iniae* and the Δ*cpsD* mutant were diluted 1:10 in a 96-well plate. Growth was monitored via optical density readings at 600 nm, in quadruplicate, every 30 min for 8 h. Hemolytic activity against sheep red blood cells was measured as described previously (19).

Invasion and adherence assays. Invasion and adherence assays were performed in collagenized 96-well tissue culture plates (Costar). White bass epithelial cells (WBE27) were seeded at a density of 1×10^5 cells per well and allowed to grow overnight. The medium was replaced with 100 μl DMEM containing 2% FBS. Bacteria from a mid-log-phase culture were diluted in DMEM with 2% heat-inactivated FBS, and 100 μl was added to achieve a multiplicity of infection (MOI) of 10 (bacteria to cells). Following centrifugation at 350 × *g* for 5 min, the plate was incubated for 1 h at 28°C with 5% CO_2. The cells were washed three times with DMEM and incubated in fresh DMEM with 20 μg/ml penicillin (Invitrogen) and 200 μg/ml of gentamicin (Invitrogen) for 2 h to kill extracellular bacteria. Cells were then washed three times with phosphate-buffered saline (PBS) and lysed by trituration in 100 μl of 0.01% Triton X-100 (Sigma). Surviving intracellular bacteria were quantified by plating serial dilutions of lysed cell supernatant on THA. Adherence assays were carried out in a similar manner except that no antibiotics were used and the bacteria were incubated with the cells for 30 min and washed five times with PBS to remove nonadherent bacteria prior to enumeration of CFU.

Capsular polysaccharide isolation and purification. Capsular polysaccharide was extracted from 2 liters of 94-426 *S. iniae* culture by using methods described for other encapsulated species (20). Briefly, overnight cultures were treated with a final concentration of 1% Cetavlon, a polycationic detergent that precipitates polyanionic polysaccharides. The precipitate was collected by centrifugation and resuspended in water, and $CaCl_2$ added to a final concentration of 1 mM to separate polysaccharide from detergent. Nucleic acids were precipitated from solution by adding 25% (vol/vol) ethanol, followed by centrifugation. Capsule in the supernatant was subsequently precipitated by ethanol at a final concentration of 80% (vol/vol). Contaminating protein, traces of Cetavlon, and other low-molecular-mass contaminants were removed with proteinase K digestion and extensive dialysis against a buffer composed of 10% ethanol, 50 mM NaCl, and 5 mM Tris. Capsule was further purified with a Sephacryl 200 gel filtration column using 50 mM ammonium formate elutions. Column fractions were tested for neutral sugar estimation by phenol sulfuric acid assay (14). Void-volume fractions were pooled and concentrated by speed vacuuming and analyzed by deoxycholate-polyacrylamide gel electrophoresis (42) and Alcian blue staining (42).

Glycosyl composition analysis. The glycosyl composition of capsular polysaccharide was determined by the preparation and analysis of trimethylsilyl methylglycosides (40). Briefly, samples were methanolyzed with 1 M methanolic HCl at 80°C for 18 h, followed by re-N-acetylation of methylglycosides by use of pyridine-acetic anhydride in the presence of methanol at 100°C for 1 h. The free hydroxyl groups of re-N-acetylated methylglycosides were trimethylsilylated using Tri-Sil reagent (Pierce) at 80°C for 20 min. The volatile trimethylsilyl methylglycosides were then analyzed by combined gas-liquid chromatography–mass spectrometry (GLC-MS) using a DB-1 capillary column (J&W Scientific) (30 m by 0.25 mm), and detection was done with a mass selective detector (Hewlett-Packard HP 5890 series II GC interfaced to a 5971A mass selective detector).

In vivo fish challenges. Groups of 20 (~40-g) HSB (*Morone chrysops* × *Morone saxatilis*) were used for in vivo infection studies. Fish were maintained at 25°C in ~75-liter flowthrough tanks. An overnight culture of each *S. iniae* strain

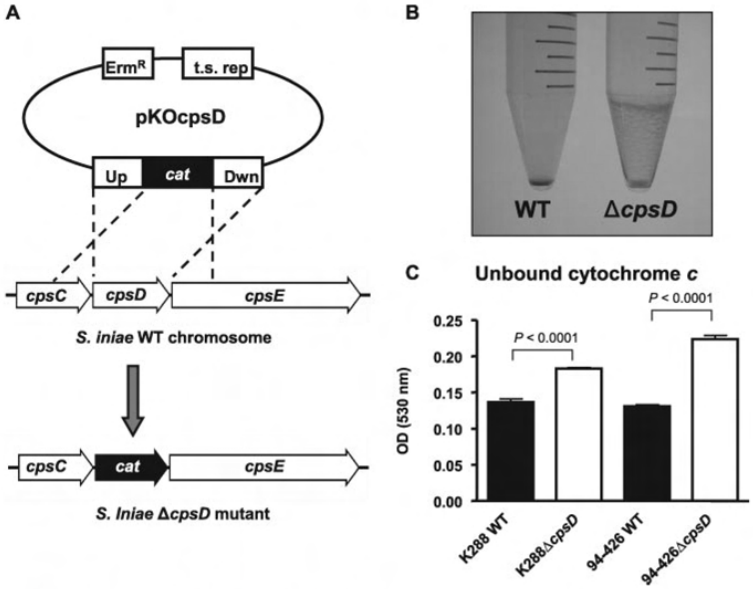

FIG. 1. Allelic exchange mutagenesis of *S. iniae cpsD* results in a capsule-deficient phenotype. (A) A knockout plasmid, pKOcpsD, was created, containing erythromycin resistance (Erm^R), a temperature-sensitive origin of replication (t.s. rep), and a chloramphenicol resistance gene (*cat*) flanked by homologous regions of DNA upstream (Up) and downstream (Dwn) of *cpsD*. The knockout plasmid was used for precise in-frame replacement of the *S. iniae cpsD* gene with *cat*. (B and C) Allelic replacement of *cpsD* resulted in reduced capsule production as seen by reduced buoyancy in liquid culture (B) and a reduction in negative cell surface charge measured indirectly through amount of positively charged cytochrome *c* remaining unbound to the bacteria (mean ± SEM) (C). OD, optical density.

was diluted 1:10 and grown to mid-log phase. The bacteria were pelleted, resuspended in PBS, and diluted appropriately to deliver the desired dose in a 100-μl intraperitoneal (i.p.) injection. Survival was monitored for 7 days. Fish challenges were carried out in an ALAAC-certified facility following IACUC-approved protocols.

Survival in whole blood. Blood was extracted via syringe from the caudal vein of three HSB and collected in a heparinized tube. Three hundred microliters of each blood sample was immediately added to two 2-ml siliconized microcentrifuge tubes with approximately 300 CFU of mid-log-phase bacteria. The tubes were incubated with shaking at 30°C for 1 h. Two 100-μl aliquots from each blood sample were spread onto THA to enumerate surviving bacteria. Survival was calculated as a percentage of remaining bacteria relative to the starting inoculum.

Total cell killing and intracellular survival. Total phagocytic survival assays were carried similarly to invasion and adherence assays. Bacteria were incubated with CLCs at an MOI of 0.1. Cells were lysed and plated as described above for invasion and adherence assays. Survival is expressed as CFU per ml of lysed cell supernatant at each time point. Intracellular growth assays were carried out in a manner similar to that for entry assays. Bacteria were incubated with the CLCs at 28°C at an MOI of 10. After 1 h, the medium was replaced with fresh DMEM with 20 μg/ml penicillin (Invitrogen) and 200 μg/ml of gentamicin (Invitrogen) to kill extracellular bacteria. After 4 h in antibiotics, the medium was replaced with fresh DMEM containing 2% FBS. The cells were lysed and plated to determine surviving CFU as described above. Survival is expressed as CFU per ml of lysed cell supernatant at each time point. For a visual comparison of phagocytosis in CLCs, bacteria were labeled by being grown to an optical density at 600 nm of 0.40 in THB plus 50 μg/ml fluorescein isothiocyanate (FITC) (Molecular Probes). Bacteria were washed twice in PBS and added at an MOI of 10 to CLC monolayers as described above. After incubation with antibiotics as described above to kill extracellular bacteria, monolayers were washed two times with PBS, and SYTOX Orange (Molecular Probes) was added to a final concentration of 0.5 μM to each well. Bacteria were visualized with a Zeiss Axiovert 100 inverted microscope with appropriate fluorescent filters. FITC-labeled intracellular bacteria appeared green, and remaining extracellular bacteria killed by antibiotics were labeled with SYTOX Orange and appeared red. Images were captured with a charge-coupled device camera using the Axiovision software package (Zeiss).

Resistance to AMPs. Mid-log-phase cultures of *S. iniae* were diluted in fresh THB to ~3×10^4 CFU/ml, and 180 μl of this bacterial suspension was added to wells of a 96-well plate. Dilutions of the antimicrobial peptides moronecidin (28) (1.5 μM final concentration in the well) and polymyxin B (Sigma) (60 μM final concentration in the well) were prepared in distilled water and added to wells in 20-μl volumes; distilled water alone was used as a control. To measure antimicrobial killing kinetics, 20-μl aliquots from each well were serially diluted in PBS and plated at specified time points after addition of the antimicrobial peptide for CFU determination. Each treatment was performed in four replicate wells. Kinetic killing data were calculated for each time point by dividing the treatment group CFU by the control CFU.

Oxidant susceptibility assay. Bacterial strains were grown to mid-log phase and diluted 1:10 in PBS, and 100 μl was added to a 96-well plate, resulting in ~3×10^6 CFU/well. Hydrogen peroxide (H_2O_2) (Fisher Scientific) was added to a 0.035% final concentration. Bacteria were incubated at 30°C, and the reaction was quenched at time end points by adding 1,000 U of catalase (Sigma). Dilutions were plated on THA to determine the number of surviving CFU.

RESULTS

Mutagenesis of *S. iniae cpsD* reduces surface capsular polysaccharide. Precise, in-frame allelic replacement of the *cpsD* gene was achieved in *S. iniae* strains K288 and 94-426 to create K288 Δ*cpsD* and 94-426 Δ*cpsD* (Fig. 1A). Each *S. iniae* Δ*cpsD* allelic replacement mutant exhibited reduced buoyancy in liquid culture (Fig. 1B). Loss of capsule was corroborated by loss of anionic charge on the surface of Δ*cpsD* mutants, as determined by decreased cytochrome *c* binding (36) (Fig. 1C). As reported for capsule mutants of other streptococci (15, 31) and also observed in our observations of increased chain length in GBS strain COH1 Δ*cpsE* isogenic capsule mutants (44) (data not shown), the *S. iniae* Δ*cpsD* mutants formed chains of

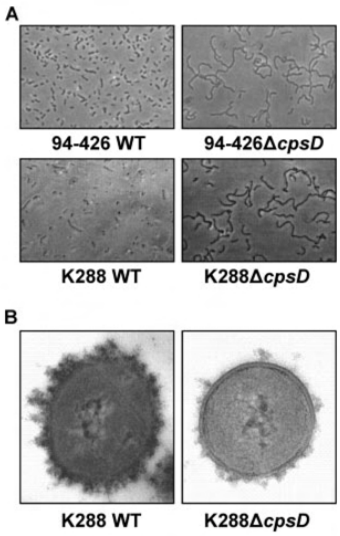

FIG. 2. Deletion of *cpsD* increases coccus chain length and reduces extracellular capsular polysaccharide. (A) Bright-field microscopy (magnification, ×400) reveals increased coccus chain length of the Δ*cpsD* mutants compared to WT *S. iniae*. (B) Transmission electron microscopy (magnification, ×15,500) shows a decrease in capsule in the K288 Δ*cpsD* mutant compared to WT K288.

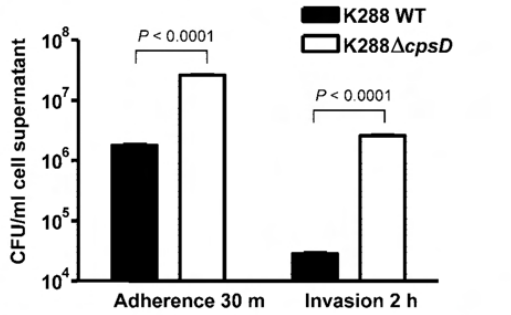

FIG. 3. Reduction in capsule increases adherent and invasive properties of *S. iniae*. *S. iniae* strain K288 was less adherent and invasive than capsule-deficient strain K288 Δ*cpsD* after incubation with WBE27 white bass epithelial cells. Adherent bacteria were enumerated after 30 min, and invasive intracellular bacteria were enumerated at 2 h (mean ± SEM). Similar results were observed for WT strain 94-426 and it Δ*cpsD* mutant.

greater length than the WT parent strains (Fig. 2A). Compared to the wild type, a clear reduction in surface capsular polysaccharide in the K288 Δ*cpsD* mutant was visualized through transmission electron microscopy (Fig. 2B). The Δ*cpsD* mutants had identical growth rates and similar hemolytic activity to the WT *S. iniae* strains (data not shown). It should be noted that complementation of the K288 Δ*cpsD* mutant was attempted by cloning the *S. iniae cpsD* gene into the multiple cloning sites of the constitutive high-expression plasmid pDCerm (25) and the tetracycline-inducible expression plasmid pLR16T (41). Expression of *cpsD* in pDCerm did not restore a wild-type phenotype; however, complementation with pLR16T (over a tight range of tetracycline levels) resulted in partial restoration of WT liquid culture buoyancy and coccus chain length phenotypes to the K288 Δ*cpsD* mutant (data not shown).

***S. iniae* capsule mutants show increased epithelial cell adherence and invasion.** A frequent observation in capsule-deficient streptococci is an enhancement of cellular adherence and invasion compared to those of WT strains (23, 35). Consistent with this pattern, the Δ*cpsD* mutants displayed a ~10-fold increase in adherence and a ~100-fold increase in intracellular invasion of cultured white bass epithelial cells compared to the parent strains ($P < 0.0001$) (Fig. 3).

***S. iniae* capsular sugars are reduced in the Δ*cpsD* mutant.** GLC-MS composition analysis of a capsular monosaccharide preparation revealed that the capsule of WT *S. iniae* strain 94-426 potentially contains L-fucose, D-mannose, D-galactose, D-glucose, D-glucuronic acid, *N*-acetyl-D-galactosamine, and *N*-acetyl-D-glucosamine (Table 1). It is possible that some of these sugars exist in noncapsular polysaccharides. We found a significant reduction in putative capsular monosaccharides, with the exception of D-mannose, in the capsule-deficient Δ*cpsD S. iniae* isogenic mutant (Fig. 4). Given its relative abundance in both WT and Δ*cpsD S. iniae*, it is possible that D-mannose exists as a noncapsular, cell surface polysaccharide component.

Reduction of capsule attenuates *S. iniae* infection in hybrid striped bass. The effect of the Δ*cpsD* mutation on *S. iniae* virulence was assessed through an i.p. infection challenge in HSB (Fig. 5A and B). Both WT *S. iniae* strains resulted in 100% HSB mortality within 1 week at an inoculum of 3×10^6 CFU. In contrast, injections of up to 100-fold-greater inocula of the respective Δ*cpsD* mutants caused no mortality or visual signs of infection in the fish.

***S. iniae* capsule promotes resistance to whole-blood and macrophage killing.** To elucidate potential mechanisms for the observed in vivo attenuation of the capsule mutants, *S. iniae* survival in fresh whole HSB blood was measured. Both of the *S. iniae* Δ*cpsD* mutants were significantly more susceptible to blood killing than the WT strains (Fig. 6A), indicating increased clearance by innate immune defenses. To further assess the role of the capsule in promoting *S. iniae* survival, WT *S. iniae* and the isogenic Δ*cpsD* mutant strains were incubated with a cultured fish macrophage cell line. The capsule-deficient mutant was over 20-fold more susceptible to killing by the macrophages in the in vitro assay ($P < 0.0001$) (Fig. 6B).

TABLE 1. Monosaccharide components of *S. iniae* strain 94-426 extracellular polysaccharide

Monosaccharide	mol % in:	
	WT	Δ*cpsD* mutant
L-Fucopyranose	7.90	2.20
D-Mannopyranose	20.26	67.50
D-Galactopyranose	24.70	10.12
D-Glucopyranose	21.60	9.12
D-Glucuronic acid	10.40	4.30
N-Acetyl-D-galactosamine	7.30	3.50
N-Acetyl-D-glucosamine	7.40	2.0

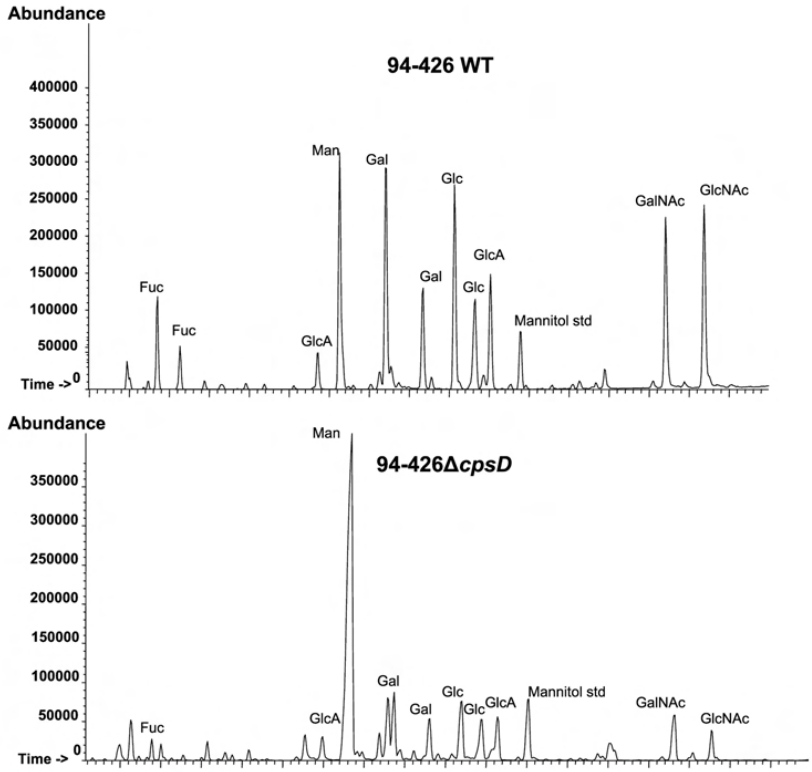

FIG. 4. GLC-MS analysis of *S. iniae* extracellular polysaccharides shows a reduction in 94-426 Δ*cpsD* monosaccharides compared to wild type. GLC-MS elution spectra after capsular preparation from on overnight culture of *S. iniae* are shown. The spectra define the potential capsular monosaccharides of *S. iniae* and indicate a significantly decreased abundance of capsular monosaccharides in the 94-426 Δ*cpsD* capsule-deficient mutant compared to the wild type. Sugar abbreviations: Fuc, L-fucose; Man, D-mannose; Gal, D-galactose; Glc, D-glucose; GlcA, D-glucuronic acid; GalNAc, *N*-acetyl-D-galactosamine; and GlcNAc, *N*-acetyl-D-glucosamine.

An *S. iniae* capsule mutant is less susceptible to cationic AMPs. One evolutionarily conserved mechanism for innate immune defense against bacterial infection is the production of cationic AMPs by phagocytes and other host cell types. We compared the susceptibilities of both WT *S. iniae* strains and the Δ*cpsD* isogenic mutants to the HSB AMP moronecidin and found the mutant strain to exhibit significantly ($P < 0.0001$) delayed killing kinetics (i.e., increased resistance) (Fig. 7A). Similar differences ($P < 0.001$) were observed in parallel assays performed with the bacterially derived cationic AMP polymyxin B (Fig. 7B). These studies indicate that the susceptibility to whole-blood and macrophage killing of the capsule-deficient strains does not derive from enhanced sensitivity to AMPs.

Loss of *S. iniae* capsule expression does not affect hydrogen peroxide sensitivity. An additional mechanism for phagocyte control of bacterial pathogens is reactive oxygen species generated through the oxidative burst. We compared the sensitivities of WT *S. iniae* strains and the isogenic mutants to killing by hydrogen peroxide and observed no biologically significant differences (Fig. 7C), suggesting that avoidance of oxidant killing mechanisms does not explain the contribution of capsule to *S. iniae* survival in the whole-blood and macrophage killing assays.

Capsular polysaccharide expression by *S. iniae* impedes phagocytotic uptake. Further investigations were performed to determine the step at which *S. iniae* capsule expression interfered with phagocyte killing, using a cultured fish macrophage cell line. The macrophages bound (Fig. 8A) and internalized (Fig. 8B) the capsule-deficient mutants much more efficiently than WT *S. iniae*. Upon phagocytosis by the macrophages, both WT and Δ*cpsD* mutant strains were rapidly and effectively killed intracellularly (Fig. 8C). Thus, the contribution of the *S. iniae* capsule to resisting phagocytic clearance was through impeding phagocytosis, not enhancing intracellular survival.

DISCUSSION

Capsule is an important extracellular feature of many bacterial species, with functions including protection against desiccation, adherence to host tissues, and resistance to both innate and adaptive host defenses (43). The extracellular polysaccharide capsules of several pathogenic streptococci have been established as virulence factors (29), acting through mechanisms including molecular mimicry (12), resistance to complement-mediated killing (13, 30), antigenic variation (5, 10), and impairment of phagocytosis (2, 47, 49). Here we used

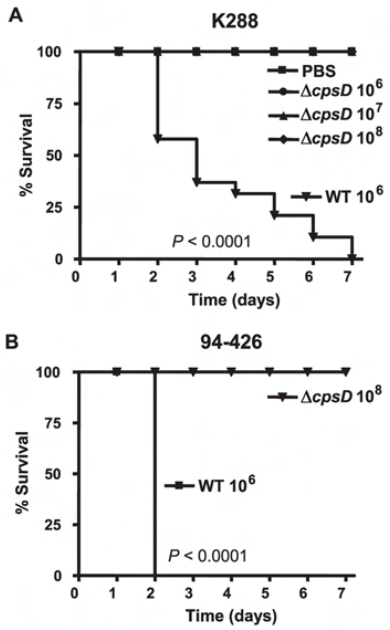

FIG. 5. Capsule contributes to *S. iniae* virulence in vivo as indicated in Kaplan-Meier survival plots showing attenuation of the Δ*cpsD* mutants. (A) Hybrid striped bass (groups of 20) were injected intraperitoneally with 3×10^6 CFU of WT K288; with 3×10^6, 10^7, or 10^8 CFU of the K288 Δ*cpsD* mutant; or with PBS. (B) Hybrid striped bass (groups of 20) were injected intraperitoneally with 3×10^6 CFU of WT 94-426 or with 3×10^6 CFU of the 94-426 Δ*cpsD* mutant. Mortality (100%) was observed only in the WT-injected fish for each strain.

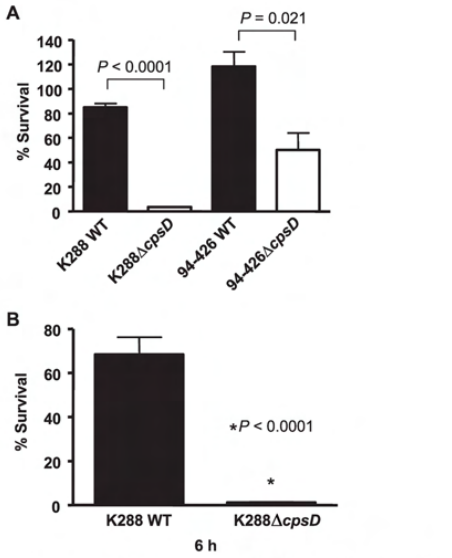

FIG. 6. *S. iniae* capsule decreases susceptibility to killing by whole blood and macrophages. (A) Survival of capsule-deficient Δ*cpsD* mutants is significantly decreased compared to that of wild-type *S. iniae* following 1 h of incubation in fresh whole fish blood. (B) K288 Δ*cpsD* is more sensitive than wild-type *S. iniae* to total phagocytic killing after a 6-h incubation with CLCs. Data are presented as mean ± SEM.

allelic replacement mutagenesis to provide evidence of a gene (*cpsD*) required for *S. iniae* capsule synthesis and a genetically defined study of the virulence role of capsule in this important leading aquaculture pathogen.

Allelic exchange mutagenesis of *cpsD* in two virulent *S. iniae* strains resulted in a capsule-deficient phenotype, with characteristics similar to those of capsule mutants of other streptococcal species, such as reduced negative cell surface charge, reduced buoyancy in liquid culture, and elongated coccus chain morphology. Transmission electron microscopy supported these observations and revealed a clear reduction in cell surface capsular polysaccharide in the Δ*cpsD* mutant compared to the wild type. Likewise, as reported for capsule-deficient GBS (24) and *Streptococcus pyogenes* (46), the *S. iniae* Δ*cpsD* mutants had increased adherence to and invasion of epithelial cells. We were unable to achieve full complementation to the wild-type phenotype of the Δ*cpsD* mutant by return of the wild-type gene on a constitutive expression plasmid. This may not be surprising, however. *cpsD* homologues in other streptococci have been shown to be essential for capsule polymerization and export to the cell surface but also have been shown to play a complex regulatory role involving protein autophosphorylation, with phosphorylation state regulating capsule production (4, 33). Complementation of this gene is likely to be difficult, and to date none of the studies addressing the function of *cpsD* in other streptococci through mutagenesis have reported successful complementation of this gene (see references 4, 11, and 33, among others). Nonetheless, the lack of clear complementation data restricts us from definitive conclusions regarding the role of *cpsD* in capsule production and leads us to hypothesize that either (i) toxicity to the bacterial cells may result from CpsD overexpression; (ii) the stoichiometry of CpsD interactions with other gene products involved in capsule biosynthesis is delicate and overexpression may decrease capsule production; or (iii) our chromosomal mutation, though by sequencing appearing to represent a precise allelic replacement from ATG start codon to stop codon, could have unanticipated polar effects elsewhere in the capsule operon, resulting in a capsule-deficient phenotype.

It is interesting to note that the capsule-deficient *S. iniae* mutants are significantly more resistant to AMPs. It is thought that charge plays a role in the binding affinity of cationic AMPs to the generally anionic bacterial surfaces. Through cytochrome *c* binding affinity assays we demonstrated that the Δ*cpsD* capsule-deficient mutants have reduced net negative surface charge compared to WT *S. iniae*, as expected with the loss of anionic capsular sugars from the cell surface. In *S. pyogenes* and *Staphylococcus aureus*, an increase in negative surface charge due to the loss of teichoic acid D-alanylation resulted in increased susceptibility to AMPs (27, 37). A similar charge-related mechanism may explain our results.

Considerable work has been done to characterize the monosaccharide sugar components of other pathogenic streptococci; however, the individual sugars and repeating multimer units of *S. iniae* capsular polysaccharides are unknown. We performed GLC-MS analysis of *S. iniae* extracellular polysaccharides and found a variety of neutral and charged sugars,

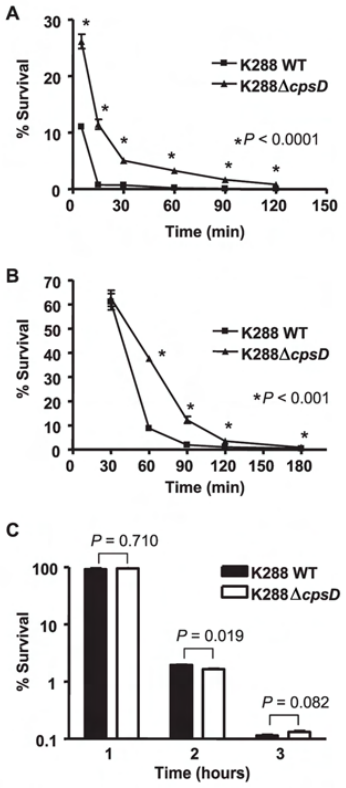

FIG. 7. Encapsulated *S. iniae* is more vulnerable to cationic antimicrobial peptides the Δ*cpsD* capsule mutants but equally susceptible to hydrogen peroxide killing. (A and B) Antimicrobial peptide kinetic killing profiles for 1.5 μM moronecidin (A) and 60 μM polymyxin B (B) indicate decreased sensitivity to AMPs for the K288 Δ*cpsD* mutant compared to wild-type K288 *S. iniae*. Similar results were observed for the 94-426 Δ*cpsD* mutant. (C) No biologically significant difference in survival of WT K288 and K288 Δ*cpsD* is observed following 1-, 2-, and 3-h incubations with hydrogen peroxide. Similar results were observed for the 94-426 Δ*cpsD* mutant. Data are presented as mean ± SEM.

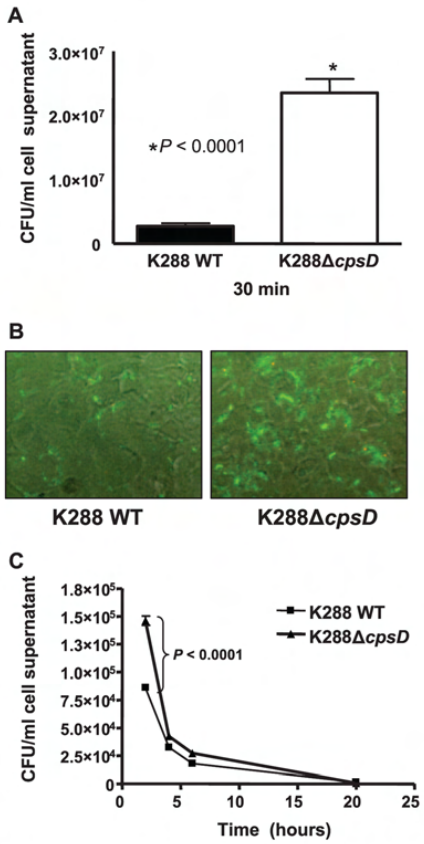

FIG. 8. Capsule hinders phagocytosis of *S. iniae* but does not confer intracellular protection against phagocytic killing. (A) The capsule-deficient K288 Δ*cpsD* mutant has increased binding affinity to the surface of CLC compared to WT K288 (mean ± SEM). (B) The K288 Δ*cpsD* mutant also is phagocytosed by CLCs more rapidly than WT K288. Fluorescence imaging (magnification, ×400) shows phagocytosed intracellular bacteria labeled with FITC (green), and adherent extracellular bacteria are labeled with SYTOX Orange (red). (C) Once phagocytosed, the K288 Δ*cpsD* mutant and WT K288 are both effectively killed over time as seen through enumeration of viable intracellular *S. iniae* in CLCs at 2, 4, 6, and 20 h postincubation (mean ± SEM).

indicating that *S. iniae* likely possesses a complex capsule structure. Based on the component sugars, capsules composed of hyaluronic acid, chondroitin, or heparin are possibilities, with the potential addition of neutral sugar side chains. Further analysis of the multimer subunits of *S. iniae* capsular polysaccharide will help to elucidate the role of *cpsD* in capsule synthesis. In GBS, CpsD functions in the later stages of capsular polysaccharide synthesis involving export of repeating units of sugars to the cell surface (11). In the *S. iniae* capsule-deficient mutants, we found intact capsular polysaccharide with all of the component sugars in roughly the same ratios; however, the amounts of these sugars were greatly reduced. In light of these data and the electron micrographs, we hypothesize that the *S. iniae* Δ *cpsD* mutants likely assemble the capsule polysaccharide repeating units but are deficient in their ability to express wild-type levels of capsular polysaccharides and export them to the cell surface.

The capsule-deficient Δ*cpsD* mutants of *S. iniae* proved to be highly attenuated in vivo, even when delivered at 100 times the 100% lethal dose for the WT. A reduction in virulence for capsule-deficient mutants has been demonstrated for several streptococcal species, including *S. suis* (49), GBS (45), and *S. pyogenes* (22, 46). In an effort to elucidate the mechanism by which capsule protects *S. iniae*, we noted that the capsule-deficient mutants were extremely sensitive to clearance in fresh HSB blood and in cell culture with fish macrophages. We documented that sensitivity to neither AMPs nor reactive oxygen species was increased in the capsule-deficient mutants. We did discover, however, a profound decrease in the ability of the capsule-deficient mutants to avoid binding and phagocytosis by fish macrophages. In *S. pneumoniae*, strains with increased negative surface charge due to the absence of choline-

binding proteins are significantly less adherent to human monocytes (50), suggesting that a major component of the increased binding affinity of capsule-deficient *S. iniae* to host cells may be related to loss of surface negative charge. Alternatively, another explanation for affinity towards host cells of unencapsulated streptococci could be the ability of capsule to mask surface-associated proteins or other factors that may play a role in host cell binding (24). Though *S. iniae* capsule potentially interferes with certain steps in the pathogenic process by decreasing attachment and invasion of epithelial cells, capsule simultaneously reduces the ability of host phagocytes to bind and phagocytose the bacterium. In our i.p. fish infection challenge, the latter phenomenon clearly plays the more critical role in determining the outcome of infection.

In summary, we present here proof that capsule is involved in *S. iniae* virulence. Through allelic replacement we have shown that *cpsD* is likely required for complete *S. iniae* capsule expression and that capsule plays a role in *S. iniae* virulence through its ability to lower the rate of phagocytosis by host immune cells. Our use of a natural host-pathogen infection challenge showed that the Δ*cpsD* mutants are over 100-fold attenuated compared to WT *S. iniae*, despite increased resistance to AMPs and increased adherence to and invasion of epithelial cells. Finally, we present preliminary data showing the individual monosaccharide components of *S. iniae* capsular polysaccharide. Having established capsule as a key *S. iniae* virulence determinant, further studies can explore the details of capsule synthesis and capsule regulation during various stages of the *S. iniae* infectious process.

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Chapter IV

Surface-anchored virulence factors: M-like protein and C5a peptidase

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PLoS one

Streptococcus iniae M-Like Protein Contributes to Virulence in Fish and Is a Target for Live Attenuated Vaccine Development

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Abstract

Background: *Streptococcus iniae* is a significant pathogen in finfish aquaculture, though knowledge of virulence determinants is lacking. Through pyrosequencing of the *S. iniae* genome we have identified two gene homologues to classical surface-anchored streptococcal virulence factors: M-like protein (*simA*) and C5a peptidase (*scpI*).

Methodology/Principal Findings: *S. iniae* possesses a Mga-like locus containing *simA* and a divergently transcribed putative *mga*-like regulatory gene, *mgx*. In contrast to the Mga locus of group A *Streptococcus* (GAS, *S. pyogenes*), *scpI* is located distally in the chromosome. Comparative sequence analysis of the Mgx locus revealed only one significant variant, a strain with an insertion frameshift mutation in *simA* and a deletion mutation in a region downstream of *mgx*, generating an ORF which may encode a second putative *mga*-like gene, *mgx2*. Allelic exchange mutagenesis of *simA* and *scpI* was employed to investigate the potential role of these genes in *S. iniae* virulence. Our hybrid striped bass (HSB) and zebrafish models of infection revealed that M-like protein contributes significantly to *S. iniae* pathogenesis whereas C5a peptidase-like protein does not. Further, *in vitro* cell-based analyses indicate that SiMA, like other M family proteins, contributes to cellular adherence and invasion and provides resistance to phagocytic killing. Attenuation in our virulence models was also observed in the *S. iniae* isolate possessing a natural *simA* mutation. Vaccination of HSB with the Δ*simA* mutant provided 100% protection against subsequent challenge with a lethal dose of wild-type (WT) *S. iniae* after 1,400 degree days, and shows promise as a target for live attenuated vaccine development.

Conclusions/Significance: Analysis of M-like protein and C5a peptidase through allelic replacement revealed that M-like protein plays a significant role in *S. iniae* virulence, and the Mga-like locus, which may regulate expression of this gene, has an unusual arrangement. The M-like protein mutant created in this research holds promise as live-attenuated vaccine.

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Introduction

Streptococcus iniae is a significant finfish pathogen responsible for annual losses in aquaculture exceeding $100 million [1]. Though originally isolated from a freshwater Amazon dolphin (*Inia geoffrensis*) [2], and capable of causing infection in elderly or otherwise immunocompromised humans [3], *S. iniae* is predominantly a fish pathogen with a broad host range of fresh and saltwater species such as trout, tilapia, salmon, barramundi, yellowtail, flounder, and hybrid striped bass (HSB) [4]. Mortality resulting from *S. iniae* is often attributed to meningoencephalitis which manifests following systemic dissemination of bacteria through the bloodstream and major organs [4]. Currently there are no commercial vaccines approved for prevention of *S. iniae* infection in US aquaculture.

Our understanding of *S. iniae* pathogenesis is limited. To date only three *S. iniae* virulence factors have been characterized in the context of fish virulence: the capsular polysaccharide which contributes to phagocyte resistance [5,6]; the cytolysin streptolysin S which contributes to host cell injury [7,8]; and phosphoglucomutase, which is required for cell wall rigidity and resistance to cationic antimicrobial peptides [9]. In each case, the identified *S. iniae* virulence determinant shared homology with counterparts expressed by other major streptococcal pathogens of humans and/or animals. In an effort to identify additional genes involved in *S. iniae* pathogenesis, we have used pyrosequencing [10] (454 Life

Sciences) of a virulent isolate to identify candidate genes sharing homology with proven virulence factors of the leading human pathogen, *Streptococcus pyogenes* (group A *Streptococcus*, GAS), a well characterized close genetic relative of *S. iniae* [11].

In GAS, many virulence genes are part of a pathogenicity regulon known as Mga (multiple gene regulator of group A *Streptococcus*) [12,13]. Mga is a "stand-alone" global gene regulator that exerts positive transcriptional regulation on downstream genes in the proximal Mga locus, and distally in the genome through binding of the Mga protein to consensus upstream promoter regions [14,15]. The most extensively studied component of the Mga regulon is M protein, a surface-anchored virulence factor [16,17] that contributes to GAS cellular adherence and invasion [18,19], resistance to phagocytic clearance [20,21], host inflammatory activation [22,23], and serotypic diversity [24,25]. Other members of the GAS Mga regulon include genes for additional M-like surface proteins and the gene encoding the C5a peptidase ScpA, a bifunctional virulence factor capable of inactivating the complement derived neutrophil chemoattractant C5a [26,27], while also contributing to GAS epithelial cell adhesion [28].

Here we identify genes *simA* and *scpI* in a virulent *S. iniae* isolate which share homology with genes encoding the GAS Mga-associated virulence factors M-like protein and C5a peptidase, respectively. We provide bioinformatic analyses of these two genes and the *S. iniae* Mga-like Mgx locus, comparing different *S. iniae* isolates and other streptococcal pathogens. Through targeted allelic replacement mutagenesis coupled with *in vitro* and *in vivo* models of *S. iniae* pathogenesis, we assess the roles of these genes as virulence determinants of this leading aquaculture pathogen, and demonstrate a key role for *simA*. Finally, we examine the utility of the Δ*simA* mutant as a live attenuated vaccine.

Results

SiMA and its relationship to other streptococcal M family proteins

The 1,566 bp M-like protein gene *simA*, from *S. iniae* strain K288, encodes a 521 amino acid gene product, SiM (*S. iniae* M-like protein), with a predicted precursor protein mass of 57.5 kDa. This M-like protein gene is identical to the recently published *simA* gene sequences from *S. iniae* strains QMA0076 and QMA0131 [29]. BLAST (tblastn) analysis groups SiMA closest to the *S. uberis* lactoferrin binding protein, Lbp (32% identity, 49% positive) [30] and the *S. dysgalactiae* subsp. *dysgalactiae* (GCS) M-like protein, DemA (31% identity, 51% positive) [31], though SiMA has near comparable similarity to a number of other streptococcal M family proteins (Fig. 1A). Amino acid sequence alignments between SiMA and related M family proteins, as expected, showed the highest degree of similarity in the C-terminus which includes the LPXTG Gram-positive surface anchor motif (Fig. 1B, S1) [32].

sim sequences are highly conserved across a diverse panel of *S. iniae* isolates

The *sim* genes from a panel of 11 *S. iniae* isolates from various hosts and geographical regions in North America were analyzed for DNA sequence similarity (Table 1). Only three of these strains (29178, 95006, and 02161A) varied from the *simA* consensus sequence defined in the wild-type (WT) K288 strain, a finding consistent with previous observations [29]. ATCC strain 29178 (freshwater dolphin abscess isolate) possesses a silent A→G single nucleotide polymorphism (SNP) in nucleotide 741, maintaining the Gln-247 residue, and is identical to the *simA* allele sequence for the QMA0140 dolphin isolate [29]. Another A→G SNP was found in strain 95006 (tilapia abscess isolate) at nucleotide 1,430, changing Gln-477 to Arg-477. The most significant *sim* sequence variation was found in a tilapia brain isolate (02161A), which possess a 40 bp insertion duplication starting at bp 595. This insertion generates a frameshift mutation splitting the gene into two potential ORFs, likely leading to severely altered or absent function. The first ORF is predicted to encode a truncated N-terminal SiM fragment of predicted 22.7 kDa mass, but would lack the LPXTG consensus motif for sortase-mediated cell wall anchoring of Gram-positive surface proteins. The second ORF would encode a C-terminal SiM fragment of 33.7 kDa containing the LPXTG motif, but would lack the hydrophobic N-terminal leader sequence involved in protein secretion.

ScpI and its relationship to other streptococcal C5a peptidase-family genes

The *S. iniae scpI* (Streptococcal C5a peptidase-like gene of *S. iniae*) gene is 3,369 bp in length and encodes a predicted 1,122 amino acid gene product with a mass of 123.3 kDa. BLAST (tblastn) analysis indicates ScpI has equal degrees of similarity (37% identity, 55% positive) to the C5a peptidases of GAS (ScpA of the Manfredo M5 strain) [33] and group B *Streptococcus* (*S. agalactiae*, GBS) (ScpB of the A909 strain) [34]. Though the proteolytic functionality of ScpI is unknown, it does contain the conserved serine protease catalytic triad of Asp-130, His-193, and Ser-512 [35]; however due to differences in overall protein size these conserved residues fall at slightly different locations in ScpI (Asp-114, His-181, Ser-501) (Fig. S2). Analysis of ScpI also indicates conservation of the C-terminal LPXTN cell surface anchor motif (Fig. S2).

S. iniae does not possess a GAS-like Mga locus

S. iniae does not possess a typical GAS-like Mga locus arrangement containing M family protein and C5a peptidase genes, where these genes in GAS are located adjacently and downstream of the *mga* gene transcribed in the same direction [15]. Unlike GAS, in *S. iniae* strain K288, the M-like protein gene (*simA*) is located adjacent to a divergently transcribed *mga*-like gene, *mgx* (Fig. 2A) and the C5a peptidase gene (*scpI*) is located elsewhere on the chromosome. The Mga-like Mgx shares almost complete amino acid similarity (98.2% identity, 98.4% positive) with the Mgx sequence reported for *S. iniae* strain QMA0076 [29]. The limited sequence variation is isolated to the C-terminal amino acids leading up to and including 7 additional amino acids found in the Mgx proteins of strains K288 and 9117 (a human isolate currently being sequenced by Baylor College of Medicine Human Genome Sequencing Center, BCM-HGSC) which extend beyond the 495 amino acid Mgx protein found in strains 02161A and QMA0076. *S. iniae* Mgx is most similar (tblastn, 39% identity, 58% positive) to the Mga-like Mgc putative regulatory protein of *S. dysgalactiae* subsp. *equisimilis* (GCS/GGS) [36].

Our sequencing efforts, as well as the BCM-HGSC 9117 genome project, indicate the presence of a chromosomal region downstream from *mgx* which encodes two ORFs with high BLAST similarity to regions of Mgx and other Mga-like regulatory proteins, potentially representing an evolutionary distant duplication of a *mga*-like gene, whose function was lost through mutations over time (Fig. 3). Strain 02161A, however, through sequence variation in this region, including a 117 bp deletion, possesses a 1,326 bp ORF which may encode a second putative *mga*-like regulatory gene, *mgx2* (Fig. 3). The 441 amino acid *mgx2* gene product, Mgx2, has a predicted mass of 51.7 kDa and is most similar (tblastn) to the Mgx protein of *S. iniae* QMA0076 (42% identity, 58% positive) [29], the DmgB Mga-like protein of GCS

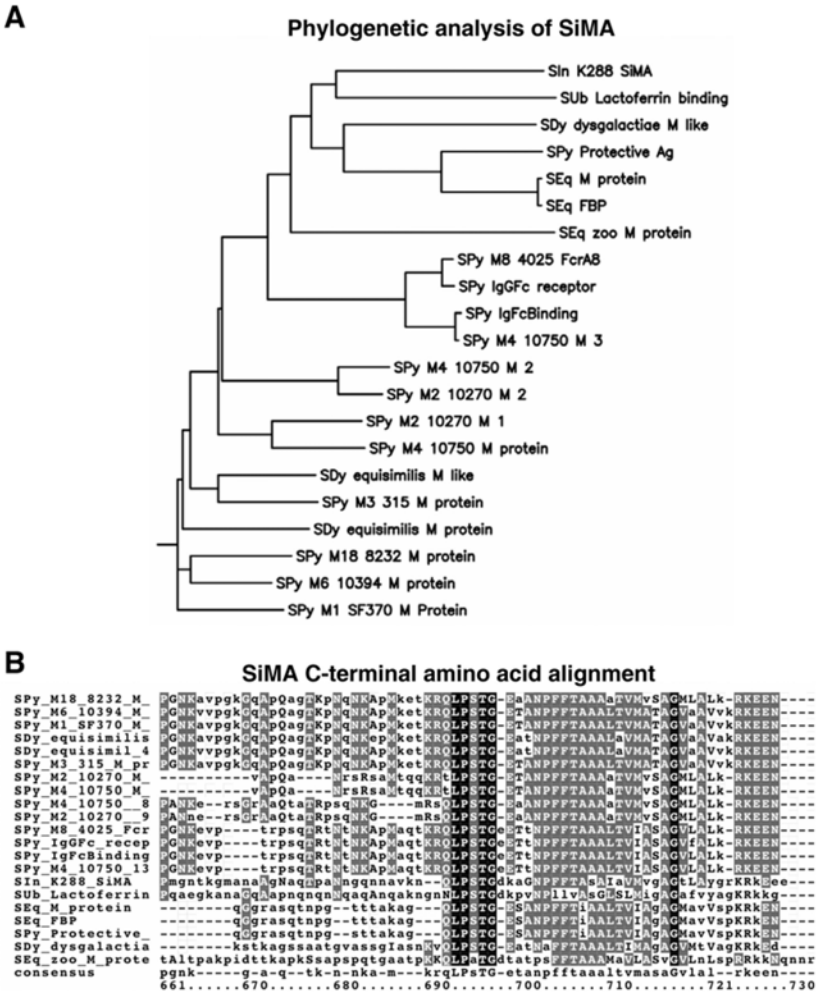

Figure 1. Bioinformatic analysis of SiMA. (A) Phylogenetic clustering of SiMA shows greatest similarity to other streptococcal M family proteins, most closely the *S. uberis* lactoferrin binding protein. (B) Amino acid sequence alignments of SiMA with other streptococcal M family proteins shows highest conservation in the C-terminal region which includes the LPXTG surface anchor motif. Strain abbreviations: SIn–*S. iniae,* SPy–*S. pyogenes,* SUb–*S. uberis,* SEq–*S. equi,* and SDy–*S. dysgalactiae.*
doi:10.1371/journal.pone.0002824.g001

strain Epi9 (32% identity, 52% positive) [31], and the Mga protein of GAS strain MGAS8232 (32% identity, 53% positive) [37].

Almost exactly halfway between the divergently transcribed *mgx* and *simA* genes (162 bp upstream from the *simA* start codon) lies a highly conserved 51 bp region, identical in isolates K288, 9117, 02161A and QMA00131 [29] (Fig. 4). This region has similarity to the established 45 bp Mga binding site for the *emm6.1* gene of M6 GAS [14] and a 47 bp region upstream of the *S. uberis* lactoferrin binding protein gene [30] (Fig. 4). Downstream from *simA* is a putative tellurite resistance protein gene, *telX*, encoding a gene product with 99% identical amino acid composition to the TelX protein of *S. iniae* strain QMA0076 [29]. The chromosomal arrangement of *mgx*, *simA*, and *telX* was identical in *S. iniae* strains K288, 9117, 02161A, and QMA0076 [29]. Aside from insertion and deletion mutations in 02161A, nucleotide level analysis of the remainder of the Mgx locus reveals high conservation between strains (Fig. 3).

Unlike GAS, but similar to the chromosomal positioning in GCS and GGS [36], the *S. iniae scpI* gene is located outside of the Mga-like locus of the chromosome (Fig. 2B) and does not possess an upstream promoter region similar to binding motifs present in Mga-regulated GAS *scpA* genes [14]. *ScpI* is bordered downstream by a divergently transcribed putative transposase (*tnpA*), a 237 bp ORF encoding a 78 amino acid gene product. TnpA has highest similarity (tblastn, 66% identity, 78% positive) within an overlapping 50 amino acid region of "IS861, transposase orfB" in the GBS A909 genome [34]. A transposase is one of the insertional elements flanking the GBS *scpB* chromosomal region and is thought to be involved in horizontal gene transfer [38]. Immediately upstream of the transposase is the phosphoglucomu-

Table 1. Information on *S. iniae* strains used in *sim* gene sequencing.

Strain	Source	Location	Host	Tissue origin	Reference
K288	KST	California	HSB	brain	[9]
K139	KST	California	HSB	brain	
K436	KST	California	HSB	brain	
94290	KST	California	HSB	internal organs	
94426	LSU	Louisiana	tilapia	brain	[5]
95006	LSU	Louisiana	tilapia	abscess	
94449	LSU	Louisiana	tilapia	abscess	
9117	UT	Ontario	human	blood	[3]
9066	UT	Ontario	fish (sp. unknown)	surface of skin	[100]
F1	UF	Florida	rainbow shark	systemic	[101]
29178	ATCC	San Francisco	freshwater dolphin	abscess	[2]
02161A	LSU	Minnesota	tilapia	brain	

Abbreviations: KST–Kent SeaTech Corporation, HSB–hybrid striped bass, LSU–Louisiana State University, UT–University of Toronto, UF–University of Florida, ATCC–American Type Culture Collection.
doi:10.1371/journal.pone.0002824.t001

tase gene (*pgmA*) which has been implicated in *S. iniae* fish virulence [9]. Upstream of *scpI* lies a 957 bp putative sugar ABC transporter gene (*satA*) with high similarity (tblastn, 90% identity, 96% positive) to the putative ABC sugar transporter SPy_1225 of GAS M1 strain SF370 [39]. The presence of *scpI* and *tnpA* in between the *satA* and *pgmA* genes in the *S. iniae* chromosome also supports horizontal transfer theories since the homologues of *satA* and *pgmA* in GAS are located adjacently in the genome [39].

Allelic replacement of *simA* and *scpI* conserves key *S. iniae* phenotypic properties

Precise in-frame allelic replacement of *simA* and *scpI* (Fig. 2A, B) generated viable mutants which retain most WT phenotypic characteristics. In particular, no differences between the Δ*simA* or Δ*scpI* mutant and the WT K288 parent strain were observed in coccoid morphology (Fig. 5A), cell buoyancy which is correlated to encapsulation (Fig. 5B), hemolytic activity against fish red blood cells (Fig. 5C), or cell surface charge (Fig. 5E). The Δ*simA* mutant did enter stationary phase at a slightly higher optical density than either the WT K288 or the Δ*scpI* mutant (Fig. 5D) and the Δ*scpI* mutant had a slightly increased frequency of multimeric cocci chains than the other two strains (Fig. 5A).

S. iniae M-like protein contributes to virulence in HSB and zebrafish infection models

Using our established *S. iniae* HSB infection model system [9] we analyzed the overall requirement of *simA* and *scpI* for fish virulence following intraperitoneal (IP) or intramuscular (IM) challenge. Compared to the WT K288 strain, the isogenic Δ*simA* mutant was completely attenuated in the HSB IP challenge ($P<0.0001$) (Fig. 6A) and caused only 10% mortality in the IM challenge group ($P<0.001$) (Fig. 6B). An IP challenge in HSB with 1,000 times the lethal WT K288 dose (3×10^8 CFU) of the Δ*simA* mutant was required to generate comparable mortality to WT K288 (data not shown). Similar to the K288 Δ*simA* mutant, *S. iniae* WT 02161A strain (with a frameshift mutation truncating the *simA*

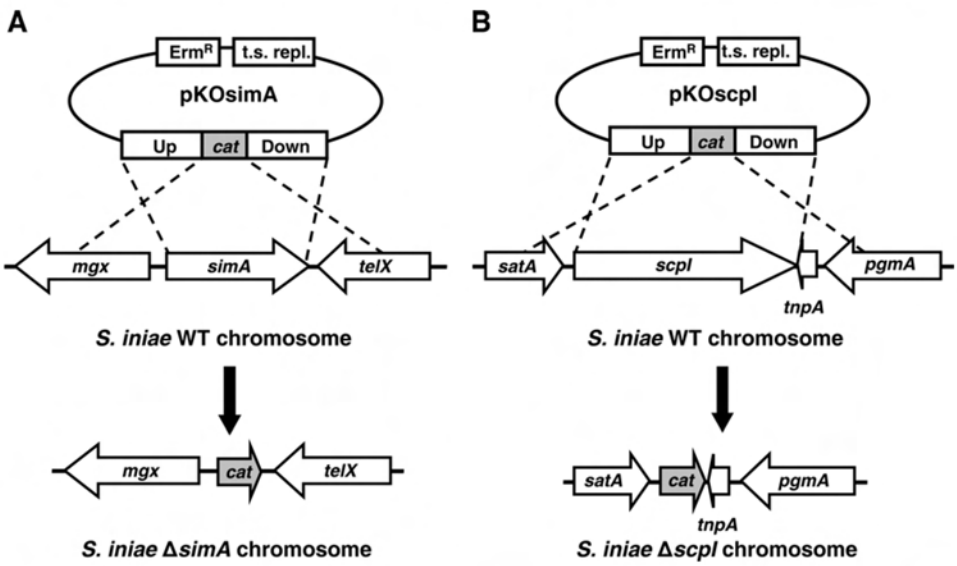

Figure 2. Allelic exchange mutagenesis of *simA* and *scpI*. Allelic exchange mutagenesis of *simA* (A) and *scpI* (B) was carried out by using knockout plasmids (pKOsimA and pKOscpI) containing ~1,000 bp flanking regions upstream (Up) and downstream (Down) nesting the *cat* gene in between. The plasmid also contains Erm resistance (Erm^R) and a temperature-sensitive origin of replication (t.s. repl.). Through two independent single crossover events, the *S. iniae simA* and *scpI* genes were precisely replaced in-frame by the *cat* gene. (A) The *simA* gene is located adjacent to a putative *mga*-like regulatory gene, *mgx*. Downstream is a divergently transcribed, putative tellurite resistance protein (*telX*). (B) The *scpI* gene lies upstream from a putative sugar ABC transporter gene (*satA*). A putative transposase (*tnpA*) flanks the downstream end of *scpI* followed by the phosphoglucomutase gene (*pgmA*).
doi:10.1371/journal.pone.0002824.g002

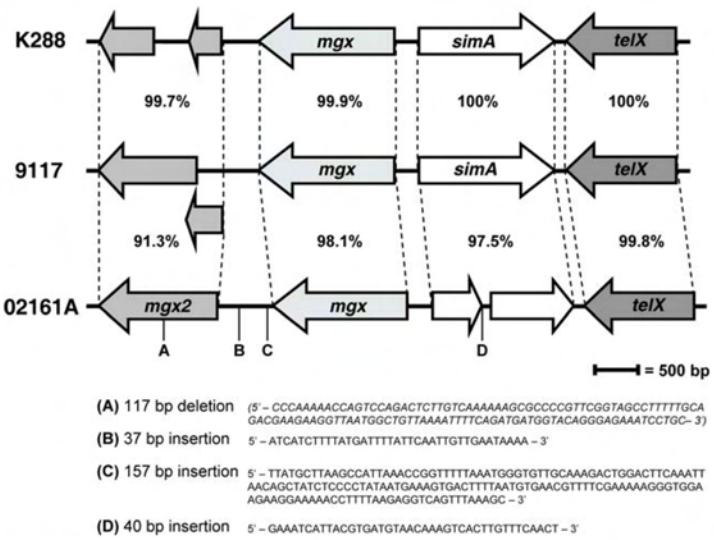

Figure 3. Nucleotide and ORF variability in the *S. iniae* Mga-like Mgx region. The *S. iniae* putative *mga*-like gene *mgx* and the putative tellurite resistance gene *telX* are highly conserved in strains K288, 9117, and 02161A. 02161A, however, has significant variation in the Mgx chromosomal region primarily due to four deletion or insertion sequences (A–D), two of which affect coding sequences. A 40 bp insertion/duplication (D) in the *simA* M-like protein gene splits it into two ORFs whose transcription and function is unknown. A 117 bp deletion (A) in the upstream *mgx* region generates a second putative *mga*-like gene, *mgx2*. In K288 and 9117 the mgx2 region is broken into two smaller ORFs. Similarity between adjacent strains is indicated as % nucleotide identity.
doi:10.1371/journal.pone.0002824.g003

ORF) was attenuated compared to strain K288 in the HSB IP challenge model ($P<0.005$) (Fig. 6A). In contrast, allelic replacement of the *scpI* gene encoding a C5a peptidase-like protein did not significantly reduce *S. iniae* virulence in the IP model ($P=0.31$) (Fig. 6A) and was actually associated with an increase in the kinetics of killing compared to WT K288 in the IM challenge model ($P<0.01$) (Fig. 6B). A zebrafish IM challenge model has also been developed for analysis of virulence factors of streptococcal pathogens [40], including the observed attenuation of a GAS C5a peptidase (ScpA) mutant compared to its parent strain [41]. We found that the *S. iniae* Δ*simA* mutant showed evidence of attenuation in this zebrafish model, producing no mortalities, though this trend did not achieve statistical significance due to low WT mortalities ($P=0.067$). Challenge with the isogenic Δ*scpI* mutant generated no evidence of attenuation and a similar mortality curve to the WT K288 *S. iniae* parent strain in the zebrafish model ($P=0.985$) (Fig. 6C). Based on the composite *in vivo* fish challenge experiments, we conclude *S. iniae* M-like protein SiMA plays a significant role in *S. iniae* invasive disease pathogenesis, while the C5a peptidase-like protein ScpI alone is not required for fish virulence upon systemic challenge by injection.

SiMA does not protect *S. iniae* against cationic AMPs

AMPs are an evolutionarily conserved innate defense mechanism [42], and likely play a role in fish resistance to bacterial infection [43]. The increased sensitivity of an *S. iniae* phosphoglucomutase mutant to cationic AMPs demonstrates the importance of *S. iniae* to protect against antimicrobial defenses [9]. To determine if enhanced AMP resistance represent a contribution of SiMA to *S. iniae* virulence, we tested the susceptibility of the Δ*simA* mutant to three AMPs: *Bacillus*-derived polymyxin B, HSB derived-moronecidin, and murine-derived CRAMP. Both WT and Δ*simA* mutant *S. iniae* strains were sensitive to all three AMPs and killed with similar efficiency: 99.10±0.03% WT vs. 99.38±0.03% Δ*simA* killing by 60 μM polymyxin B in 120 min; 99.21±0.06% WT vs. 98.74±0.14% Δ*simA* killing by 1.5 μM moronecidin in 15 min; 99.98±0.05% WT vs. 99.92±0.47%

Figure 4. Comparison of putative Mga-like binding motifs upstream of *sim* genes. The 51 bp upstream regions of *S. iniae sim* genes with high similarity to GAS *emm* gene Mga binding sites are identical in strains K288, 9117, and 02161A. A 47 bp sequence sharing similarity to Mga-like binding sites located upstream of the gene encoding the *S. uberis* lactoferrin binding protein (Lbp, a close phylogenetic relative of SiMA) is also included for comparison. *S. iniae* and *S. uberis* putative binding motifs are aligned with the established 45 bp Mga binding site found in M6 GAS upstream of the *emm6.1* M protein gene. Abbreviations: SIn–*S. iniae*, SUb–*S. uberis*, SPy–*S. pyogenes*.
doi:10.1371/journal.pone.0002824.g004

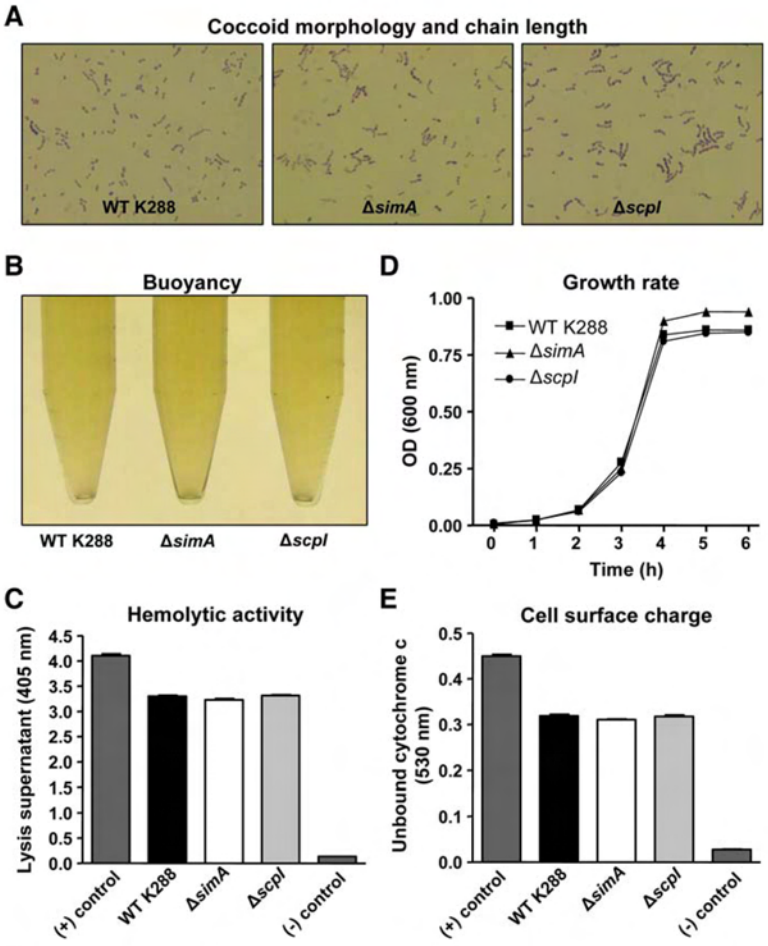

Figure 5. Basic phenotypic properties of *S. iniae* are highly conserved following allelic replacement of *simA* and *scpI*. (A) Cocci chain morphology was observed under light microscopy (Crystal Violet staining viewed under an oil immersion 100× objective). (B) General buoyancy characteristics of the strains were observed in overnight cultures grown in 15 ml conical tubes. (C) Hemolytic activity was measured through the optical density of the supernatant following incubation of HSB red blood cells with bacteria. (D) Growth rate was measured optically every 45 min in 5 ml tube cultures. (E) Bacterial cell surface charge was indirectly measured through the absorbance of unbound, positively charged cytochrome *c*, following incubation with bacteria.
doi:10.1371/journal.pone.0002824.g005

Δ*simA* killing by 16 μM CRAMP in 30 min. We conclude that SiMA does not likely contribute to relative resistance of *S. iniae* to cationic AMPs.

SiMA contributes to *S. iniae* adherence to and invasion of fish epithelial cells

The ability to adhere to and invade epithelial layers is proposed to play a role in *S. iniae* virulence [44]. We used cultured monolayers of the white bass epithelial cell line WBE27 to assess the adherence and intracellular invasive properties of *S. iniae* strains *in vitro* [45]. Compared to the WT parent strain K288, the *S. iniae* Δ*simA* mutant demonstrated significantly less adherence to (~40% reduction, $P<0.005$) and invasion (~20% reduction, $P<0.02$) of WBE27 cells (Fig. 7A, B). The levels of adherence and invasion associated with the *S. iniae* WT 02161A strain (harboring a frameshift/truncation mutation in the *simA* gene) had a similar trend ($P=0.0067$, $P<0.0001$, respectively) to those of the *S. iniae* K288 Δ*simA* mutant (Fig. 7A, B).

SiMA contributes to *S. iniae* macrophage resistance

Another described virulence property of *S. iniae* is its ability to resist phagocytosis and survive within fish leukocytes [46]. To determine if M-like protein SiMA promotes bacterial survival when exposed to phagocytic cells, a killing assay with the carp macrophage cell line CLC was performed. The survival of the Δ*simA* mutant in the presence of macrophages was similar to that of the parent strain at early time points (2 h and 4 h), however by 18 h survival of the Δ*simA* mutant was reduced over 2 logs compared to WT K288 ($P<0.0001$) (Fig. 7C). The WT 02161A strain possessing the frameshift/truncation mutation in the *simA* gene also showed significantly diminished survival compared to WT strain K288 by the 18 h time point ($P<0.0001$) (Fig. 7C).

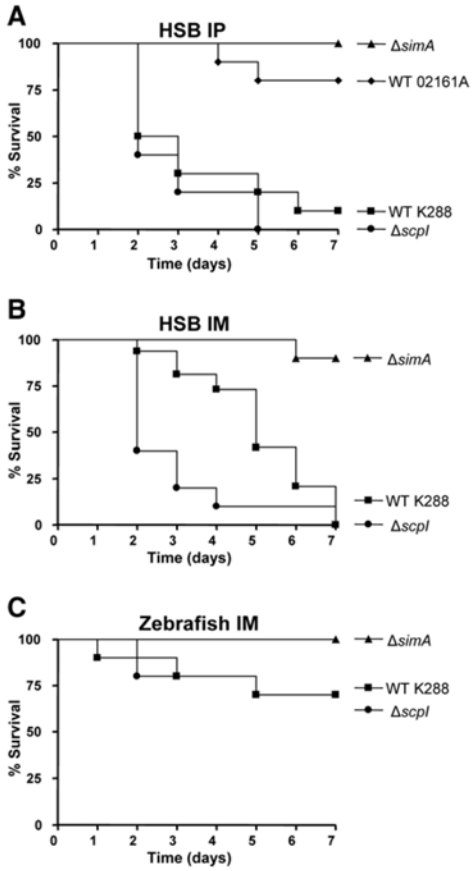

Figure 6. M-like protein contributes to *S. iniae* virulence in HSB and zebrafish infection models. (A) Juvenile hybrid striped bass (HSB) (n = 10) were injected IP with 3×10^5 CFU of WT K288 *S. iniae*, the Δ*simA* and Δ*scpI* isogenic mutants, or WT 02161A (possesses a natural frameshift mutation in *simA*). (B) Juvenile HSB (n = 10) were injected IM with 3×10^5 CFU of WT K288 *S. iniae*, or the Δ*simA* and the Δ*scpI* mutants. (C) Adult zebrafish (n = 10) were injected IM with 4×10^5 CFU of WT K288 *S. iniae* or the Δ*simA* and the Δ*scpI* mutants.
doi:10.1371/journal.pone.0002824.g006

The attenuated Δ*simA* mutant confers adaptive immune protection against *S. iniae* infection

Previous work demonstrated the effectiveness of an attenuated *S. iniae* phosphoglucomutase mutant to protect HSB against subsequent challenge with WT *S. iniae* [9]. Because the Δ*simA* mutant shows significant attenuation in our HSB infection challenges, we investigated its potential to serve as a live attenuated vaccine. IP vaccination of HSB with two different doses of the Δ*simA* mutant (3×10^4 and 3×10^6 CFU) resulted in 8% mortality in each group. No mortality was observed in the PBS mock vaccination group. Following a holding period of 90 days (~1,400 degree days), both vaccinate groups were completely protected from a lethal dose (LD_{96}, 5×10^5 CFU) of WT K288 *S. iniae* (Table 2), demonstrating the high protective capacity of this mutant as a live vaccine candidate.

Discussion

To further understanding of *S. iniae* virulence, we used whole genome pyrosequencing to identify and characterize the *S. iniae* homologues of two well-established, Mga-regulated GAS virulence factors, M-like protein (*simA*) and C5a peptidase (*scpI*). We identified in *S. iniae* strain K288 a *mga*-like locus containing the M-like protein gene *simA* and a putative *mga*-like regulatory gene *mgx*, identical in arrangement to a locus recently described in *S. iniae* strain QMA0076 [29]. The GAS Mga locus contains several downstream virulence genes regulated by Mga, including genes encoding M-proteins and C5a peptidase. Though *S. iniae* does possess a putative tellurite resistance protein gene (*telX*) downstream of *sim* (which may potentially have a role in virulence) there are no typical GAS Mga locus-like candidate virulence genes in the Mgx locus aside from *sim*. Also of note is that unlike the GAS Mga locus (and Mga-like loci in GCS/GGS), *mgx* is transcribed divergently from M-protein homologue *sim*, similar to the chromosomal juxtaposition of the closely related *S. uberis* lactoferrin binding protein gene and its putative *mga*-like regulator [30]. Additionally, the C5a peptidase-like gene (*scpI*) is positioned distally on the chromosome from *simA* and *mgx*, a chromosomal arrangement more similar to that of GCS and GGS than GAS [36]. The presence of two adjacent Mga-like genes (one of which has been disrupted with mutations in some strains) is a unique property of *S. iniae* and may hold clues to the evolution of Mga-family genes in this species. Gene duplications in the Mga locus are thought to account for the diversity of GAS M family genes [47], though we have not found any reports of duplications in *mga* or

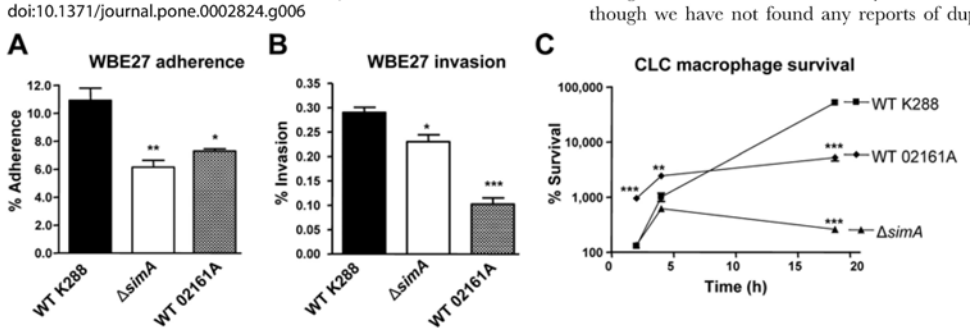

Figure 7. M-like protein contributes to *S. iniae* adherence to and invasion of cultured fish epithelial cells and resistance to killing by fish macrophages. (A) Adherence and (B) invasion characteristics of WT K288, the isogenic Δ*simA* allelic mutant, and the naturally M-deficient WT 02161A *S. iniae* strain for the fish epithelial cell line WBE27. (C) Survival of WT K288, WT 02161A, and the Δ*simA* mutant upon co-incubation with CLC fish macrophage/monocytes for 2, 4, or 18 h. Significance indicated as: * $P<0.05$, ** $P<0.005$, *** $P<0.0005$. Data are presented as mean±SEM from two-tailed t-tests.
doi:10.1371/journal.pone.0002824.g007

Table 2. Immune protection conferred by the Δ*simA* mutant in HSB.

Vaccination group	% Survival: Ä*simA* vaccination	% Survival: WT K288 challenge
3×10^4 CFU	92	100
3×10^6 CFU	92	100
PBS control	100	4

doi:10.1371/journal.pone.0002824.t002

mga-like genes. The functionality of *mgx2* in strain 02161A, and whether the mutations leading to a disrupted *simA* gene and the creation of a second putative *mgx* gene represent an alternative virulence strategy in this strain, are interesting areas for investigation. Sequence upstream of *simA* with strong similarity to the Mga transcriptional regulatory binding domains of GAS M proteins suggests that Mgx regulation of *simA* expression is likely to occur in *S. iniae*. This hypothesis is strengthened by predicted structural analysis of Mgx that indicates helix-turn-helix domains [29], a feature present in DNA-binding Mga and Mga-like regulatory proteins [48].

C5a peptidase has highly specific endoproteolytic activity against the complement system polymorphonuclear leukocyte chemotaxin C5a [26,27], thereby altering neutrophil trafficking to the site of infection [49,50]. C5a peptidase also acts as an adhesin in GBS through binding to host fibronectin [51,52] and in GAS through fibronectin independent binding [28]. Allelic replacement of the *S. iniae scpI* gene encoding a predicted C5a peptidase-like surface protein did not significantly attenuate virulence in our analyses using HSB and zebrafish infection models. While Scp inhibition of leukocyte chemotaxis is well documented in other streptococci [26,27], inactivation of this gene does not always translate into a reduction in overall *in vivo* virulence [53–55]. Teleosts do possess a potent complement system [56,57], a functional C5a homologue [58,59], and a corresponding receptor [60,61], so it is plausible that a fish pathogen would target components of this pathway. Additionally, *S. iniae* may possess other gene encoded determinants with functional redundancy to ScpI, masking virulence effects in our challenge systems. For example, in our genomic sequence analysis we also identified a putative C3 proteinase (data not shown) which may serve to inactivate the complement system upstream of C5a peptidase.

It appears that there is not a high degree of variation among SiM proteins. Sequencing a panel of 11 diverse *S. iniae* isolates generated only one significant sequence variation. We report an insertional frameshift mutation of the *simA* gene in *S. iniae* strain 02161A that splits the coding region into two smaller ORFs of unknown function, and note that this strain is attenuated in the HSB model. In GAS, frameshift mutations followed by compensatory mutations that bring the gene back into frame are proposed to play a role in antigenic variation of M proteins [62], a scenario that may play out over time in 02161A to generate a novel *sim* allele. The only other documented *sim* allele, *simB*, was found in strain QMA0141 as part of a similar comparative analysis of *sim* sequences [29]. These findings contrast the extreme variation in allele types for M-protein in GAS [63]. The functional implications of this conservation among *sim* alleles warrant further investigation. Additional sequencing efforts are needed to gauge the degree of *S. iniae* M-like protein sequence divergence and to determine if this surface protein may contribute as a serotyping determinate.

M family proteins have been shown to play a prominent role in colonization through adherence in multiple host pathogen systems [18,19]. In GAS, adherence mediated by M family proteins is not universal to all cell types, but has been shown to be particularly important in binding to keratinocytes [64] and Hep-2 cells [65]. The ability to invade non-immune cell types has also been linked to the GAS M protein [66]. Consistent with these roles we observed a decrease in adherence and invasion of the white bass epithelial cell line by the *S. iniae* Δ*simA* mutant.

A primary function function of M-like proteins involves resistance to phagocytic clearance mechanisms [67–69]. Through binding to serum proteins such as immunoglobulins, fibrinogen, and the complement regulator, factor H, M family proteins can effectively avoid phagocytosis through prevention of complement deposition. M proteins have also been shown to confer intracellular protection against phagocytic killing [21]. Similarly for SiMA, in the presence of fish macrophages, we observed over a 2 log-fold reduction in survival in the Δ*simA* mutant compared to WT K288. Our findings confirm a role for SiM in evading phagocytosis, as suggested in studies linking SiM with fibrinogen binding [29].

Our live attenuated vaccine development approach contrasts typical M protein vaccine strategies which use the protein itself or fragments thereof as the immunogen. Such vaccination strategies for the GAS M protein have required multimeric vaccines to ensure protection against a panel of relevant serotypes [70,71]. The generation of an autoimmune response through production of cross reactive antibodies [72,73] against M proteins that demonstrate molecular mimicry of host tissues [74,75] has also been a significant hurdle to GAS protein based vaccine development efforts. Whether either of these is issues is a concern in for SiM is unknown, but by deleting the M-like protein from *S. iniae* and relying on other key antigenic epitopes, both of these potential issues are circumvented in our live vaccine approach. Live attenuated vaccines also offer the advantage of prolonged, unaltered antigen presentation which can stimulate a more robust humoral and cell-mediated immune response, resulting in greater adaptive immune protection compared to inactivated bacterins or subunit vaccines in fish [76–78]. Successful demonstrations of live vaccines have been employed for a number of bacterial finfish pathogens [79–81] including *S. iniae* [9]. Though limited mortality was observed in our vaccinations with the Δ*simA* mutant, further attenuation of this strain by targeted gene disruption of additional proven virulence determinants will likely be required to provide an optimal safety profile.

In sum, through sequence analysis of the *S. iniae* genome we have identified two putative homologues of classic surface-anchored streptococcal virulence determinants, M-like protein and C5a peptidase. Allelic replacement of these two genes and analyses using our models of bacterial pathogenesis revealed that M-like protein plays a significant role in *S. iniae* virulence whereas C5a peptidase-like protein does not. Future research will investigate the regulation of these genes and their specific protein-ligand interactions. The M-like protein mutant created in this research holds promise as live attenuated vaccine. Subsequent vaccination studies will test alternative delivery options and the long-term efficacy of the Δ*simA* mutant as a live attenuated vaccine in aquaculture.

Materials and Methods

Bacteria strains, culture, transformation, and DNA techniques

The WT virulent *S. iniae* strain K288, isolated from the brain of a diseased HSB at the Kent SeaTech (KST) aquaculture facility in Mecca, CA [9], served as a background for generation of the

Δ*simA* and Δ*scpI* isogenic mutants. Additional *S. iniae* isolates used for comparative DNA sequence analysis are listed in Table 1. *S. iniae* was grown at 30°C (unless otherwise stated) in Todd-Hewitt broth (THB, Hardy Diagnostics) or on THB agar (THA). Enumeration of colony-forming units (CFU) was done through serial dilution of samples in PBS and plating on THA. β-hemolytic activity was assessed on sheep blood agar (SBA) plates (tryptic soy agar with 5% sheep red blood cells). For all assays, overnight cultures of *S. iniae* were diluted 1:10 in fresh THB and grown to mid-log phase ($OD_{600} = 0.40$). *S. iniae* strains were rendered electrocompetent for transformation through growth in THB media containing 0.6% glycine following procedures described for GBS [82]; transformants were propagated at 30°C in THB with 0.25 M sucrose. Antibiotic selection was achieved with chloramphenicol (Cm) at 2 µg/ml or erythromycin (Erm) at 5 µg/ml. *Escherichia coli* used in cloning were grown at 37°C (unless otherwise stated), shaking, under aerobic conditions in Luria-Bertani broth (LB, Hardy Diagnostics) or statically on agar (LA). When necessary, *E. coli* were grown in antibiotics: ampicillin (Amp) at 100 µg/ml, spectinomycin (Spec) at 100 µg/ml, Erm at 500 µg/ml, or Cm at 20 µg/ml. Mach 1 chemically-competent *E. coli* (Invitrogen) and electrocompetent MC1061 *E. coli* used in transformations were recovered through growth at 30°C in S.O.C. media (Invitrogen). A PureLink™ Quick Plasmid Miniprep Kit (Invitrogen) was used to isolate plasmids propagated in *E. coli*. *S. iniae* genomic DNA was isolated using a Colony Fast-Screen™ Kit (EPICENTRE Biotechnologies) or an UltraClean DNA Isolation Kit (MoBio).

Cell lines and culture conditions

The adherent CLC carp monocytic/macrophage cell line (European Collection of Cell Cultures no. 95070628) and the WBE27 white bass embryonic epithelial cell line (ATCC no. CRL-2773) [83] were grown at 28°C with 5% CO_2. Cells were maintained in 125-ml tissue culture flasks in DMEM media (Gibco) containing 10% heat-inactivated fetal bovine serum (FBS, Gibco).

Allelic exchange mutagenesis

Allelic exchange mutagenesis of *simA* (Fig. 1A) and *scpI* (Fig. 1B) with a chloramphenicol resistance gene, *cat*, was carried out as previously described for *S. iniae* [7]. A list of primers used to generate and confirm the allelic replacement mutants is provided (Table 3). PCR was used to amplify ~1,000 bp of *S. iniae* chromosomal DNA fragments directly upstream and downstream of *simA* (primers 4+5, 6+7) and *scpI* (primers 10+11, 12+13), with primers adjacent to each gene constructed to possess 25 bp 5′-extensions corresponding to the 5′- and 3′- ends of the chloramphenicol acetyltransferase (*cat*) gene from pACYC [84], respectively. The upstream (Up) and downstream (Down) PCR products were then combined with a 660-bp amplicon of the complete *cat* gene (generated with primers 1+2) using fusion PCR (primers 4+7 for *simA*, 10+13 for *scpI*) [85]. The resultant PCR amplicon containing an in-frame substitution of *simA* and *scpI* with *cat* was subcloned into the Gateway entry vector pCR8/GW/TOPO (Invitrogen) and transformed into Mach 1 *E. coli* (Invitrogen). Plasmid DNA was extracted and a Gateway LR recombination reaction was performed to transfer the fusion PCR amplicon into the corresponding Gateway entry site of a temperature-sensitive knockout vector pKODestErm (a derivative of pHY304 [86] created for Gateway cloning), thereby generating the knockout plasmids pKOsimA and pKOscpI. The knockout constructs were introduced into WT K288 *S. iniae* by electroporation. Transformants were identified at 30°C by Erm selection then shifted to the nonpermissive temperature for plasmid replication (37°C). Differential antibiotic selection (Cm^R and Erm^S) was used to identify candidate allelic exchange mutants. Targeted in-frame replacement of both genes was confirmed unambiguously by PCR reactions (primers 3+8 for *simA*, 9+14 for *scpI*) documenting the desired insertion of *cat* and absence of *simA* and *scpI* sequence in chromosomal DNA isolated from the final isogenic mutants, Δ*simA* and Δ*scpI*.

Identification of M-like protein and C5a peptidase homologues

Short contigs generated from pyrosequencing (454 Life Sciences) of the *S. iniae* K288 genome were assembled using the Phred/Phrap/Consed suite (http://www.phrap.org/phredphrap-consed.html), resulting in 1865 contigs ranging in size from 51 bp to 22 kb. Without the need of further assembly, we used these contigs to build our *S. iniae* genome database that we used for BLAST searches. Using a local version of BLAST (version 2.2.14) [87], BLAST analysis of each contig against GAS M1 (GenBank

Table 3. Primers used to generate and confirm Δ*simA* and Δ*scpI* allelic mutants.

Number	Primer name	Sequence (5′–3′)
1	catF	atggagaaaaaaatcactggatataccacc
2	catR	ttacgccccgccctgccactcatcgcagta
3	simA−1063F	aggcagagaacatttcagacaag
4	simA−1015F	agtctgtttcaaacttgtcatg
5	simA−22cat27R	ggtatatccagtgatttttttctccatgtttagggttctccttatttttc
6	cat635simA+25F	gcgatgagtggcagggcggggcgtaagctttcccttgcaacctttttcatag
7	simA+1016R	aagtacaaggatgctagccctg
8	simA+1152R	agatttcgggcaagctgccgttg
9	scpI−859F	agcagatcacattgttagtg
10	scpI−821F	tagcaccttcattagcagtc
11	scpI−18cat27R	ggtatatccagtgatttttttctccataatatattcctccaatag
12	cat635scpI+19F	gcgatgagtggcagggcggggcgtaaatcaaaaaaagaatgttcg
13	scpI+1044R	acaaaaattgctgagagttatg
14	scpI+1176R	tcttattggaacttatctgg

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Accession No. NC_002737) and M3 (GenBank Accession No. NC_004070) genomic sequences revealed the presence of putative M-like protein and C5a peptidase homologues. The contigs possessing hits for M-like protein and C5a peptidase genes were analyzed using Vector NTI software (Invitrogen) to assign open reading frames. Single-primer PCR [88] was used to sequence out from the contig ends in order to generate complete target gene sequences and provide at least 1,000 bp of flanking genomic sequence for use in allelic exchange mutagenesis. Finally, genomic regions containing *simA* and *scpI* were resequenced using standard BigDye sequencing techniques (Eton Bioscience Inc.) to confirm data generated in the initial sequencing efforts. Results from our K288 genomic analysis were compared with preliminary *S. iniae* (strain 9117) sequence data obtained from the BCM-HGSC website (http://www.hgsc.bcm.tmc.edu).

Public reporting of sequence data

Sequences for *simA* and surrounding chromosomal genes for *S. iniae* strains K288 and 02161A were deposited in the GenBank database under accession numbers EU693238 and EU714186, respectively. Sequences for *scpI* and flanking genes in strain K288 were deposited under the accession number EU693239.

Bioinformatic analyses

The amino acid sequences of all proteins were retrieved from the National Microbial Pathogen Data Resource (NMPDR) database (http://www.nmpdr.org/) by the use of the SEED similarity tool [89] and the NMPDR bidirectional best-hit engine [90]. For confirmation and completion of any missing sequences, the procedure was repeated by the use of BlastP and tBlastN algorithms [87] to search the non-redundant protein database (nr proteins) filtered to the genus *Streptococcus*. SignalP version 3.0 algorithm was used to screen the proteins sequences for Gram-positive leader peptides [91]. Several tools were used for motif finding, including InterPro [92], Pfam [93], in addition to FigFam [94] FASTA-formatted protein sequences were used as an input for the ClustalW software [95,96] available as a part of the Biology Workbench Server, (http://workbench.sdsc.edu) [97]. Phylogenetic distances of the alignment results were calculated by Phylip analysis [98], and phylogenetic trees were drawn by DrawGram [97].

Red blood cell hemolysis

Fresh, heparinized, whole HSB blood was diluted 1:1 with HBSS (no Ca^{2+} or Mg^{2+}) and 8 ml added to the top of a layered Percoll (Sigma) gradient containing 8 ml of 1.06, 1.07, and 1.08 g/ml solutions. The tube was centrifuged at RT for 30 min at 350×*g*. Red blood cells were taken from the bottom of the 1.08 g/ml density layer, washed three times in 20 volumes of PBS, and resuspended as a 2% solution (v/v). In a 96-well round bottom plate, mid-log cultures of bacteria were aliquoted in quadruplicate in volumes of 100 µl. Each well then received 100 µl of the 2% fish blood solution. Background lysis was measured in wells containing only blood cells and THB. Complete lysis was measured by wells containing blood cells, sterile THB, and 2 µl of Triton X-100. Plates were incubated at 30°C for 2 h then at 4°C for 2 h. Following centrifugation at 1,500×*g* for 5 min, 100 µl from each well was added to a new flat-bottom 96-well plate and the optical density was read at 405 nm in a microplate reader (Molecular Devices).

Cell surface charge

In triplicate, overnight cultures of each *S. iniae* strain were diluted 1:10 and grown to mid-log phase. Five ml of each culture was washed once in PBS prior to resuspension in 400 µl of MOPS buffer (pH 7.0). Next, 100 µl of a 5 mg/ml solution of cytochrome *c* (Sigma) was added. The solution was mixed thoroughly and incubated at room temp for 15 min. The bacterial suspension was pelleted (16,000×*g* for 5 min) and 200 µl of the supernatant was added to new flat-bottom 96-well plate. Controls included MOPS alone and MOPS with the same proportionate amount of cytochrome *c*. The amount of unbound cytochrome *c* was determined by absorbance of the supernatant at 530 nm.

HSB virulence challenges

In vivo virulence attenuation of Δ*simA* and Δ*scpI* mutants was assessed in juvenile (~15 g) HSB (*Morone chrysops*×*Morone saxatilis*) as previously described [7]. Groups of 10 fish per treatment group were injected intraperitoneally (IP) or intramuscularly (IM) in the dorsal muscle with 3×10^5 CFU suspended in 50 µl of PBS. Fish were maintained at 24°C in aerated, 113-l flow-through tanks and monitored one week for survival. All Fish challenges were carried out in an AAALAC-certified facility following IACUC-approved protocols.

Zebrafish virulence challenges

Adult zebrafish (*Danio rerio*, strain EKW) were challenged IM (n = 10 fish per treatment group) with 5×10^4 CFU of Δ*simA*, Δ*scpI*, or WT K288 as previously described [40]. Mid-log phase bacteria were diluted in PBS and injected in 10-µl volumes using a 0.3 cc syringe with a 29-gauge needle. Fish were maintained at 28°C in recirculated, 10-l aquariums. Survival was monitored for one week post challenge.

Antimicrobial peptide (AMP) killing assays

AMPs moronecidin [43], polymyxin B (Sigma), and CRAMP [99] were diluted in distilled H_2O to 15, 600, or 160 µM, respectively. In a 96 well round bottom plate 10 µl of each AMP solution was added to 90 µl of THB containing ~1×10^5 CFU bacteria taken from a mid-log phase culture. The plate was incubated at 30°C. At each time point a 25-µl aliquot was removed, serially diluted in THB, and plated on THA. Survival was calculated by dividing surviving CFU from each time point by the starting CFU for each strain.

Invasion and adherence assays

Invasion and adherence assays were performed in collagen-coated 96-well tissue culture plates (Nunc) using confluent monolayers of WBE27 white bass epithelial cells. Mid-log phase bacteria were centrifuged at 3,500×g for 5 min then washed once in PBS. Bacteria were then resuspended in DMEM containing 2% FBS and added to each well in 100 µl volumes to achieve a multiplicity of infection (MOI) of 5 (bacteria:cells). Following centrifugation at 350×*g* for 10 min, the plate was incubated for 60 min at 28°C with 5% CO_2. The cells were then washed twice with DMEM containing 2% FBS and incubated in fresh DMEM with 30 µg/ml penicillin (Invitrogen) and 300 µg/ml of gentamicin (Invitrogen) for 60 min to kill extracellular bacteria. Cells were then washed twice with DMEM containing 2% FBS and lysed by trituration in 100 µl of 0.025% Triton X-100 (Sigma). Surviving intracellular bacteria were quantified by plating serial dilutions of lysed cell supernatant on THA. The 30 min adherence assays were carried out in a similar manner except that no antibiotics were used and wells were washed five times with DMEM containing 2% FBS to remove non-adherent bacteria prior to trituration and enumeration of CFU.

Macrophage survival assay

Monolayers of carp macrophages (CLC) were grown as described for the invasion and adherence assays. Bacteria were

washed once in PBS then diluted in DMEM containing 2% FBS, added to the cells at an MOI of 0.05, and incubated at 28°C for 2, 4, or 18 h. Without washing, 25 µl of a 0.125% Triton X-100 solution was added to each well (0.025% final concentration). Cells were lysed and bacteria were plated as described above for invasion and adherence assays. Survival was calculated as a percentage of the input inoculum.

HSB vaccine trials

Live attenuated vaccine challenges of Δ*simA* were carried out similar to the HSB virulence studies described above. Single groups of 25 HSB (~21 g) were fin clipped to indicate treatment group and injected IP with a 100 µl volume containing 3×10^4 or 3×10^6 CFU of the Δ*simA* mutant or with PBS alone. Fish were held at 24°C for 2 weeks in 113-l aerated, flow-through tanks. Fish were cohabitated in a 1,071-l recirculating tank and held at 14-16°C for 1400 degree days (~90 days total). Fish were then sorted by treatment group into 113-l challenge tanks and acclimated to 24°C over a period of 2 days. Each group was then challenged with a 100-µl IP injection of 5×10^5 CFU of WT *S. iniae*. Survival was monitored for 2 weeks.

Statistical analyses

Data analyses were performed using the statistical tools included with GraphPad Prism 5 (GraphPad Software, Inc.). *In vitro* assay data were analyzed using unpaired two-tailed t-tests. Fish infection survival data were analyzed using a Logrank Test. $P<0.05$ was considered statistically significant. *In vitro* assays were repeated three times (with equivalent results), in quadruplicate, and data presented (mean±standard error of the mean, SEM) are from a single representative assay. *In vivo* fish challenges were repeated twice with equivalent results and data from a single experiment are shown.

Supporting Information

Figure S1 Full length amino acid sequence alignment among SiMA and M family proteins with highest similarity. Strain abbreviations: SIn-*S. iniae*, SPy-*S. pyogenes*, SUb-*S. uberis*, SEq-*S. equi*, and SDy-*S. dysgalactiae*
Found at: doi:10.1371/journal.pone.0002824.s001 (24.65 MB TIF)

Figure S2 Amino acid alignment of ScpI with GAS and GBS C5a peptidases. ScpI shows high sequence similarity to the closest C5a peptidase homologues from GAS (ScpA, SPy Manfredo M5 strain) and GBS (ScpB, SAg A909 strain). ScpI possesses the conserved LPXTN Gram-positive surface anchor motif (dark line) as well as the Asp-His-Ser catalytic triad residues (asterisks), though proteolytic function of ScpI is unknown.
Found at: doi:10.1371/journal.pone.0002824.s002 (13.22 MB TIF)

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Author Contributions

Conceived and designed the experiments: JBL MRV VN JB. Performed the experiments: JBL MRV. Analyzed the data: JBL RKA VN JB. Contributed reagents/materials/analysis tools: RKA MRV JB. Wrote the paper: JBL RKA VN JB.

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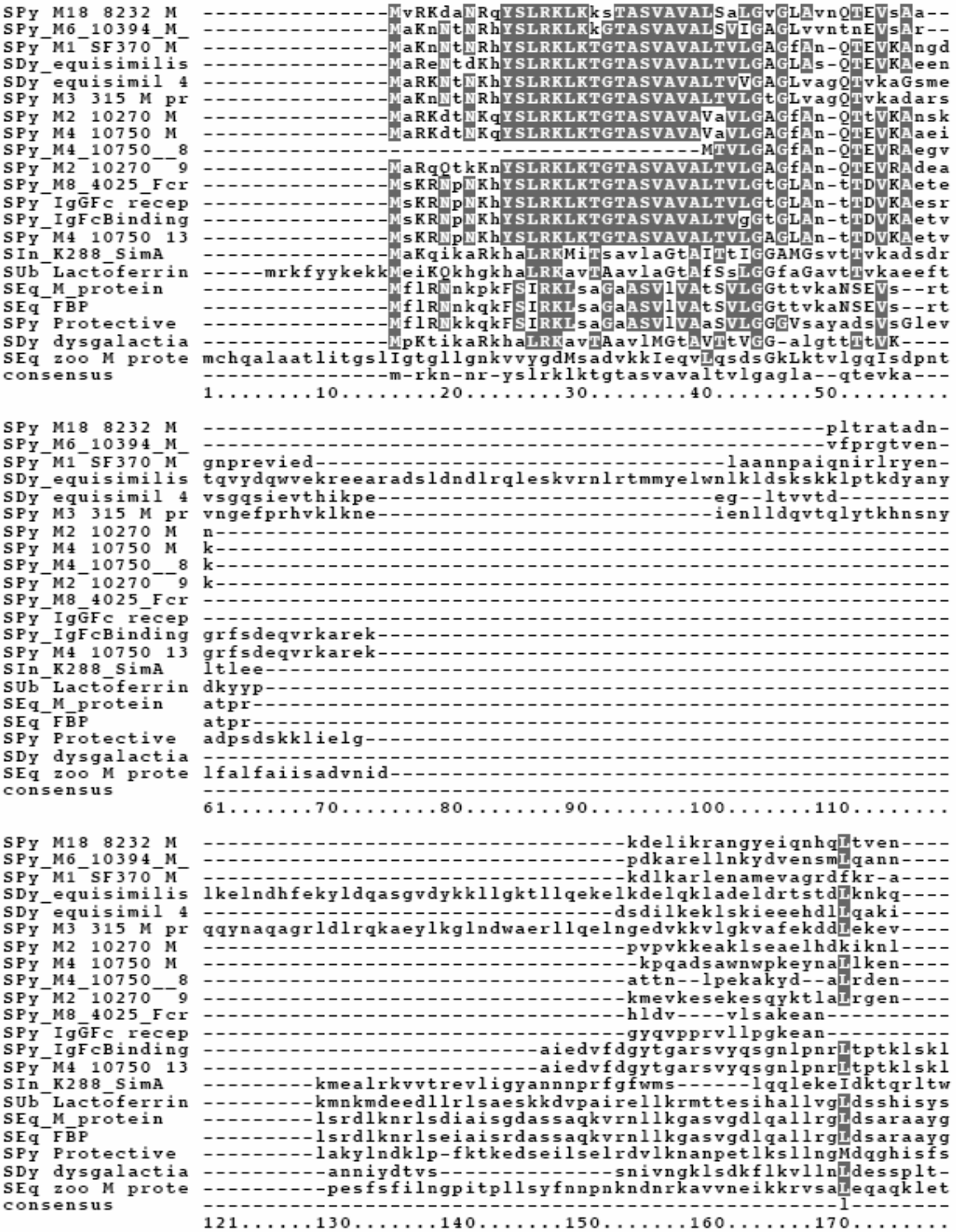
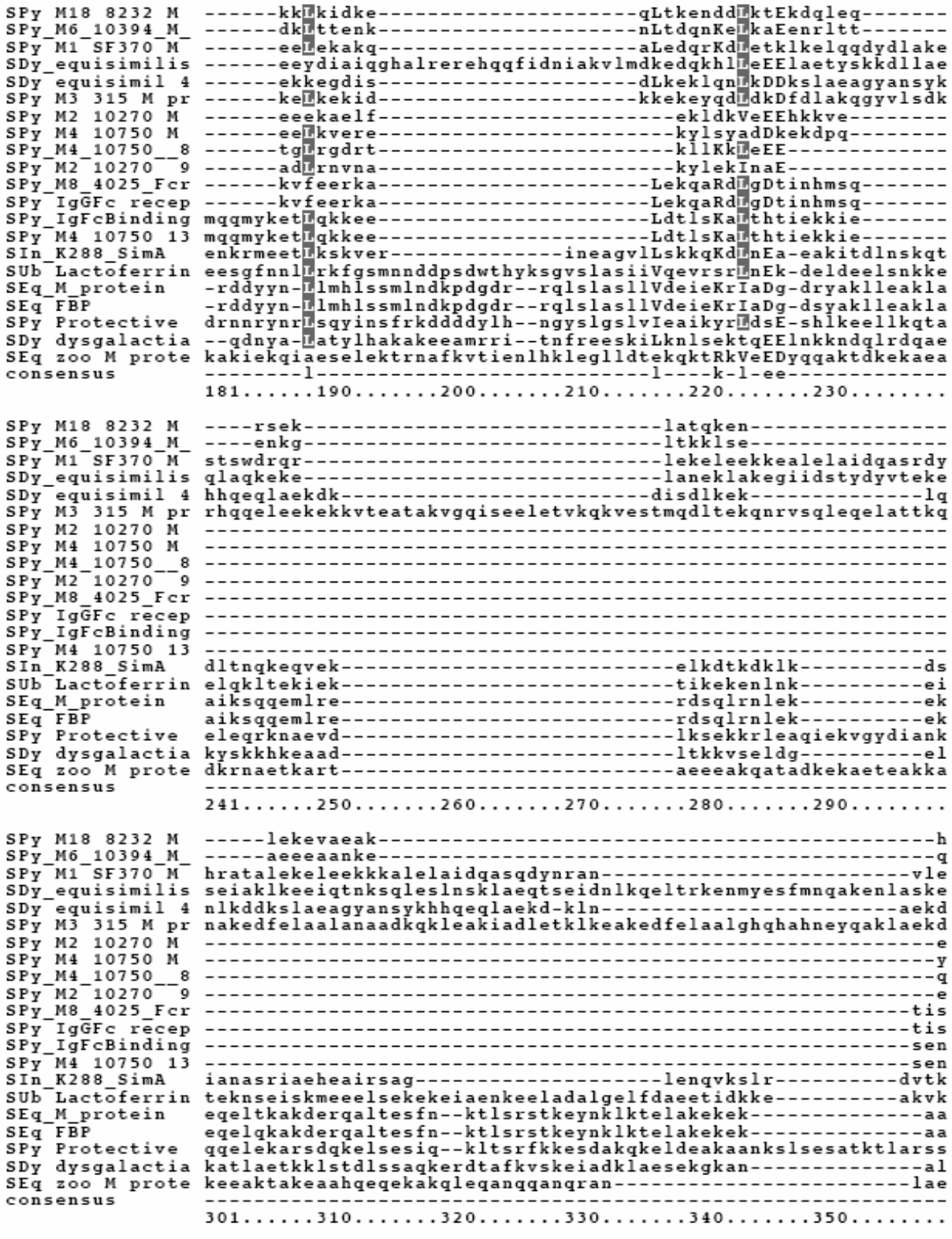
Figure S1

Alignment of SiMA with similar streptococcal M family proteins

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SDy_equisimilis
SDy equisimil 4
SPy M3 315 M pr
SPy M2 10270 M
SPy M4 10750 M
SPy_M4_10750__8
SPy M2 10270 9
SPy_M8_4025_Fcr
SPy IgGFc recep
SPy_IgFcBinding
SPy M4 10750 13
SIn_K288_SimA
SUb Lactoferrin
SEq_M_protein
SEq FBP
SPy Protective
SDy dysgalactia
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consensus
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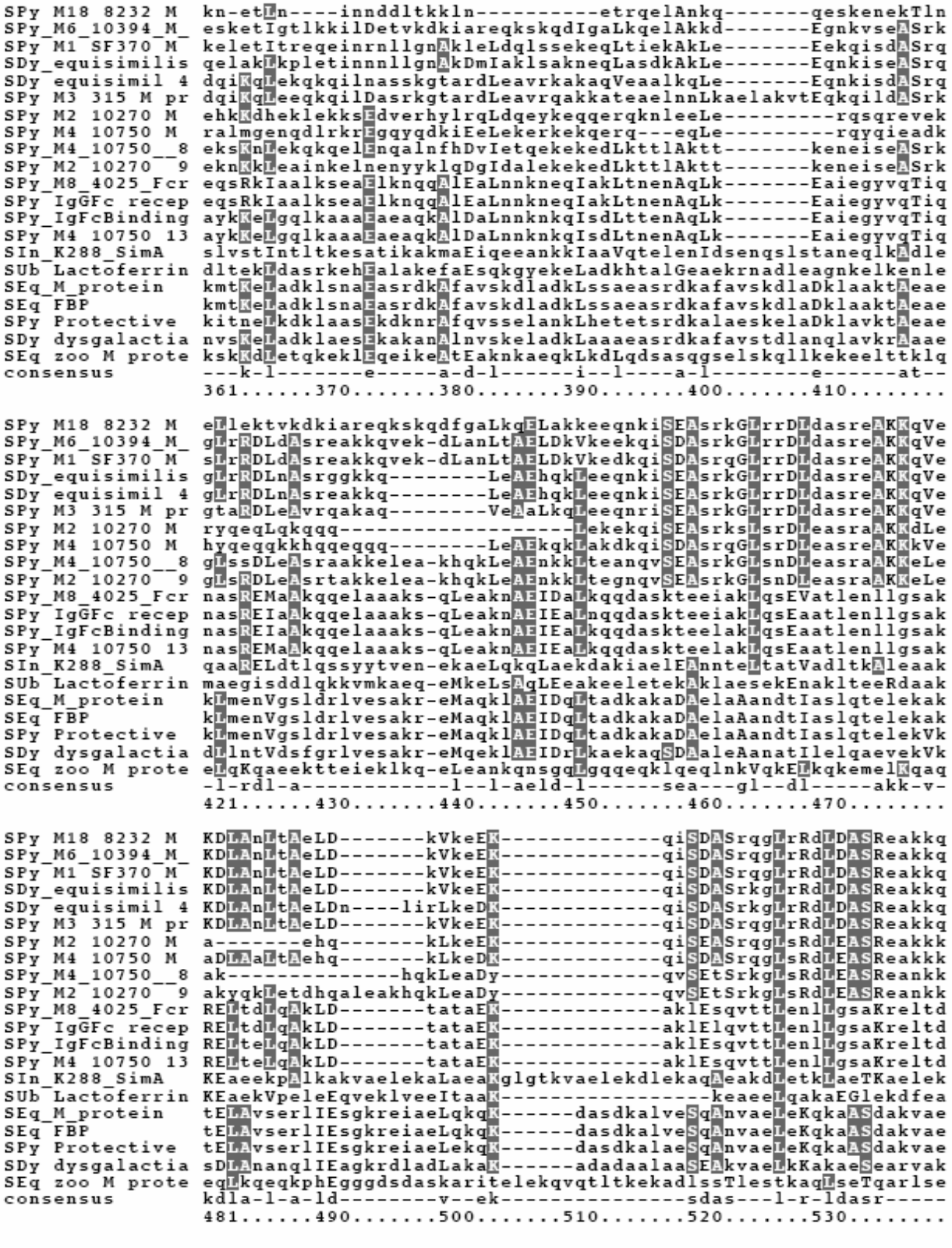
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SDy equisimil 4
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SPy M4 10750 M
SPy_M4_10750__8
SPy M2 10270 9
SPy_M8_4025_Fcr
SPy IgGFc recep
SPy_IgFcBinding
SPy M4 10750 13
SIn_K288_SimA
SUb Lactoferrin
SEq_M_protein
SEq FBP
SPy Protective
SDy dysgalactia
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consensus
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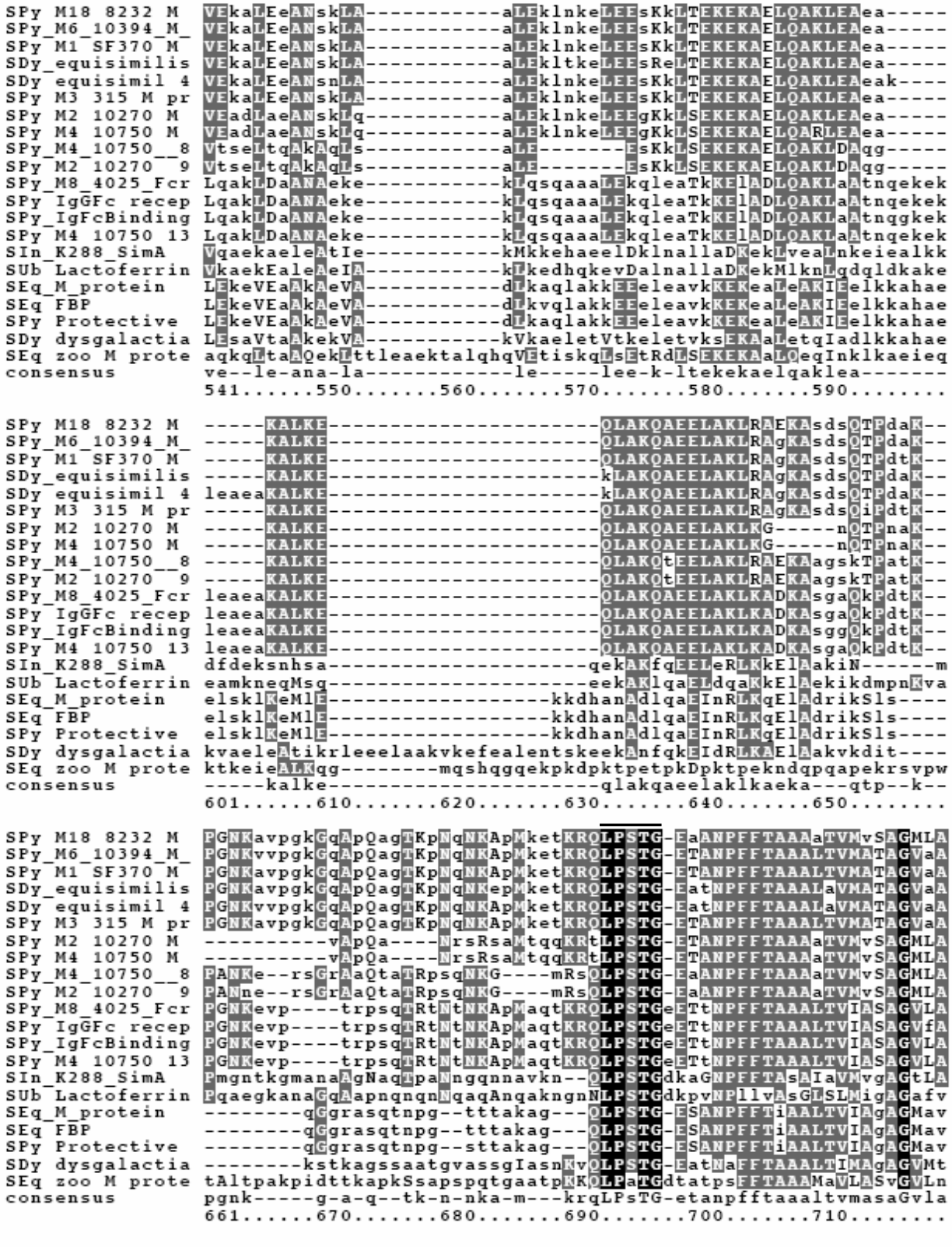
481.....490.....500.....510.....520.....530.....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SDy equisimil 4
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SPy M4 10750 M
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SPy M2 10270 9
SPy_M8_4025_Fcr
SPy IgGFc recep
SPy_IgFcBinding
SPy M4 10750 13
SIn_K288_SimA
SUb Lactoferrin
SEq_M_protein
SEq FBP
SPy Protective
SDy dysgalactia
SEq zoo M prote
consensus
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SPy_M6_10394_M_ VvkRKEEN----
SPy M1 SF370 M VvkRKEEN----
SDy_equisimilis VakRKEEN----
SDy equisimil 4 VvkRKEEN----
SPy M3 315 M pr VvkRKEEN----
SPy M2 10270 M Lk-RKEEN----
SPy M4 10750 M Lk-RKEEN----
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SPy M2 10270 9 Lk-RKEEN----
SPy_M8_4025_Fcr Lk-RKEEN----
SPy IgGFc recep Lk-RKEEN----
SPy_IgFcBinding Lk-RKEEN----
SPy M4 10750 13 Lk-RKEEN----
SIn_K288_SimA ygrKRkEee---
SUb Lactoferrin yagKRkkg----
SEq_M_protein VspKRkEN----
SEq FBP VspKRkEN----
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SDy dysgalactia VagKRkEd----
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consensus l--rkeen----
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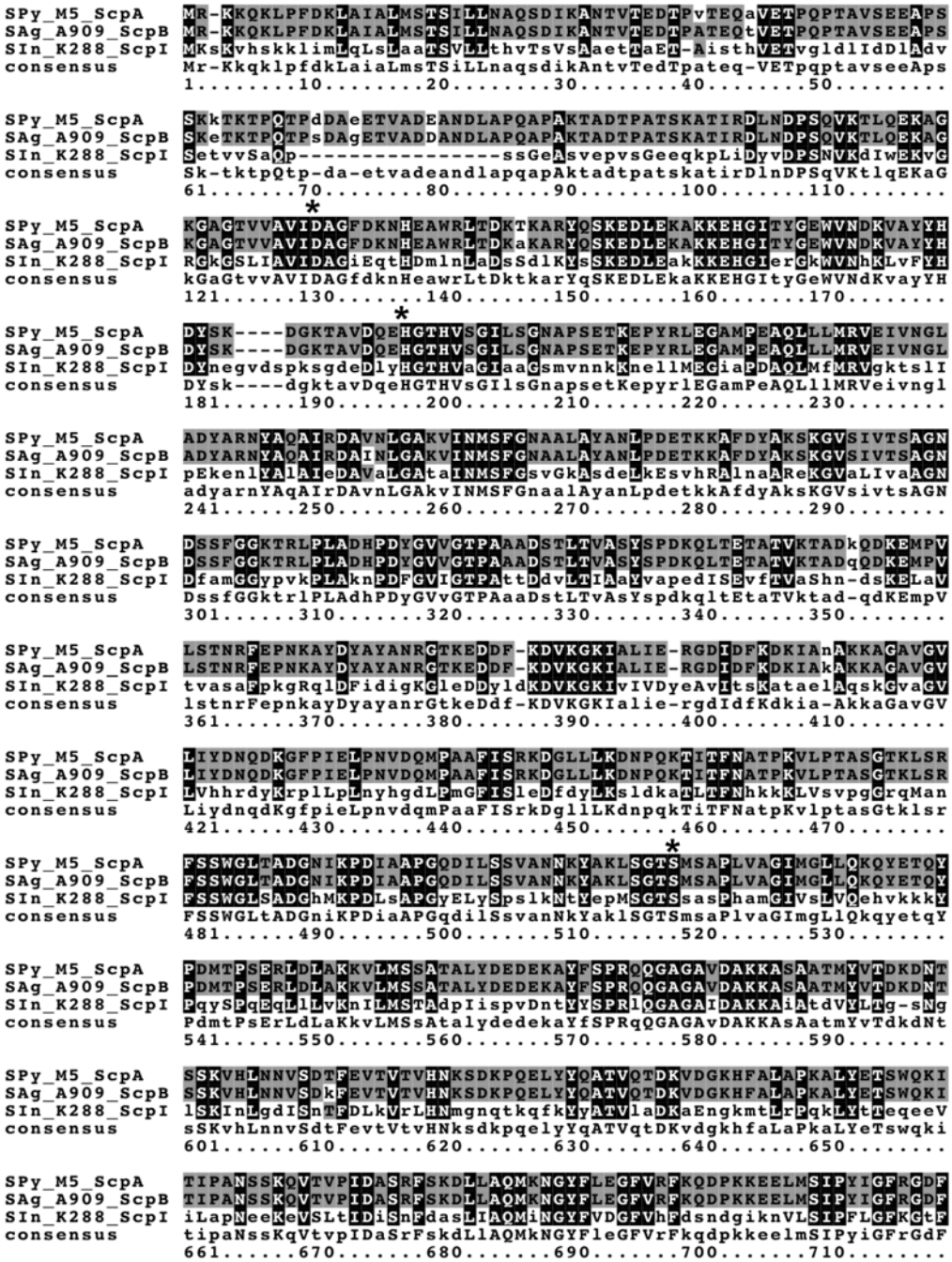
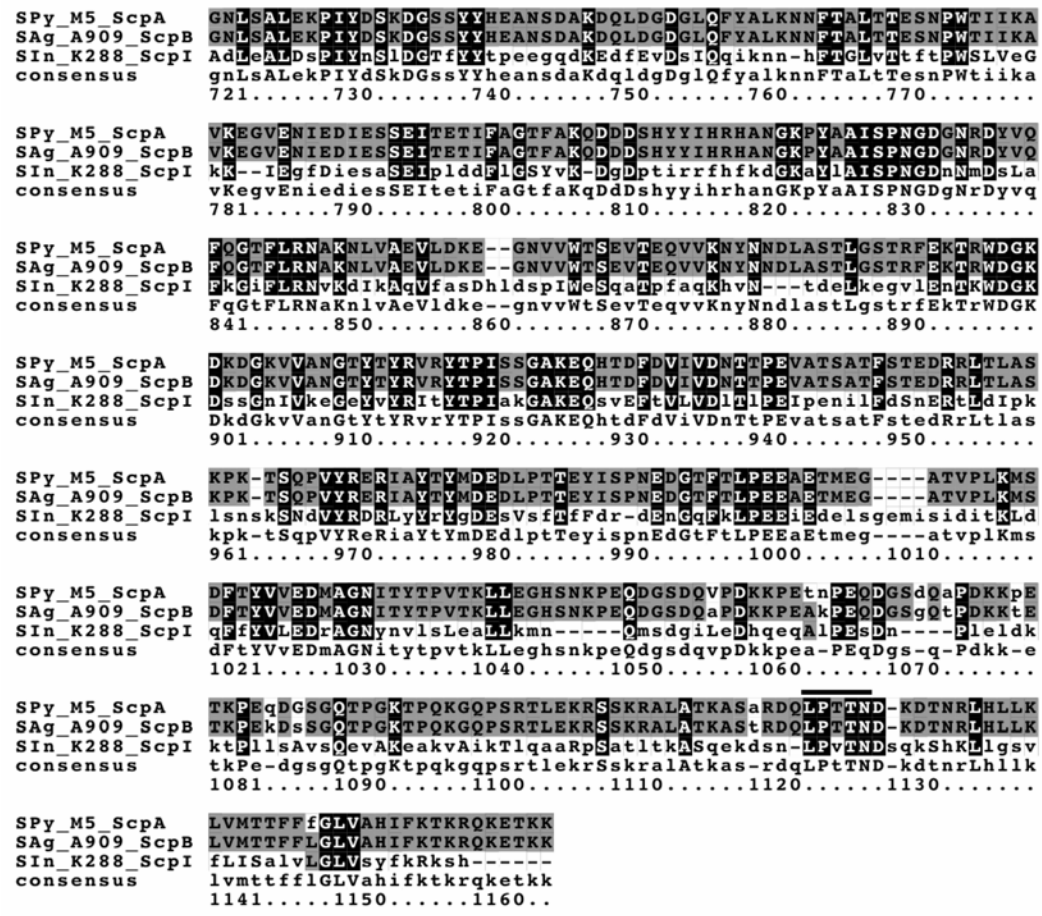
Figure S2

Alignment of ScpI with C5a peptidases from GAS and GBS

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SAg_A909_ScpB GNLSALEKPIYDSKDGSSYYHEANSDAKDQLDGDGLQFYALKNNFTALTTESNPWTIIKA
SIn_K288_ScpI AdLeALDsPIYnSlDGTfYYtpeegqdKEdfEvDsIQqiknn-hFTGLvTtftPWSLVeG
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1141.....1150.....1160..

Chapter 4 is a full reprint of the publication: Locke, J.B., Aziz, R.K., Nizet, V., and J.T. Buchanan. 2008. *Streptococcus iniae* M-like protein contributes to virulence in fish and is a target for live attenuated vaccine development. *PLoS ONE*. 3(7): e2824, with permission from all coauthors.

Chapter V

S. iniae virulence factor mutants as live attenuated vaccine candidates

Abstract

Streptococcus iniae poses a serious threat to finfish aquaculture operations worldwide. Stringent regulatory standards limit the use of antibiotics to treat *S. iniae* infections and standard killed bacterin vaccination strategies have been met with limited success. Here we investigate the potential of an alternative vaccination approach, the use of live attenuated vaccines. Three attenuated *S. iniae* strains with genetic mutations resulting in deficiency of genes involved in production of virulence factors: capsular polysaccharide (Δ*cpsD*), M-like protein (Δ*simA*), and phosphoglucomutase (Δ*pgmA*), were evaluated in parallel with an adjuvanted, formalin-killed, whole-cell *S. iniae* bacterin. Juvenile hybrid striped bass (HSB) were vaccinated through intraperitoneal (IP) injection or bath immersion and held for 800 degree days prior to challenge with a lethal dose (LD_{73}) of the isogenic, virulent wild-type (WT) *S. iniae* strain. The Δ*cpsD*, Δ*pgmA*, and bacterin vaccines provided the highest level of vaccination safety (0% mortality), though the Δ*simA* mutant (the only strain to cause some initial mortalities in vaccinated fish: 16% IP, 12% immersion), was the only vaccine candidate to provide 100% protection in both IP and immersion delivery models. Our studies further demonstrate the efficacy of live attenuated vaccines for prevention of *S. iniae* infection and show how superior performance through immersion delivery may make live vaccines an attractive option for use in commercial aquaculture settings.

Introduction

Streptococcus iniae was first isolated from an Amazon freshwater dolphin (*Inia geoffrensis*) in the 1970s (22) and since then a wide variety of both fresh and saltwater finfish have demonstrated susceptibility to infection. *S. iniae* outbreaks predominantly occur in intensive aquaculture operations and affect many commercially important fish species, including tilapia, salmon, trout, yellowtail, barramundi, flounder, and hybrid striped bass (HSB) (1). Common clinical symptoms of *S. iniae* infection include loss of orientation, lethargy, ulcers, exophthalmia, and erratic swimming (6). Mortality resulting from *S. iniae* infection is often attributed to meningoencephalitis (severe infection and inflammation of the brain) (6) and is responsible for losses exceeding $100 million annually in aquaculture (25). Despite its prevalence as an aquatic pathogen, therapeutic options for *S. iniae* are limited.

While vaccines have been a viable therapeutic strategy to prevent infections caused by many bacterial fish pathogens (28), attempts to develop *S. iniae* vaccines have been met with limited success in part due to a general lack of efficacy over extended time periods (1) and the re-emergence of serotypic variants (2). The predominant vaccination approach for *S. iniae* utilizes whole-cell, killed bacterins (7, 15) composed of autogenous isolates from aquaculture facilities experiencing outbreaks. This strategy inherently limits the breadth of coverage conferred and often serotypic variants emerge and dominate quickly (1). There are currently two whole-cell killed commercial *S. iniae* vaccines Norvax® Strep *Si* and AquaVac™ Garvetil™ (Intervet/Schering-Plough Animal Health), but neither is approved for use in the US and both are still undergoing long-term and multi-species evaluation.

Transposon and targeted mutagenesis techniques have facilitated the development of live attenuated vaccines as an alternative vaccination strategy for *S. iniae* (5, 16). The attractiveness of live attenuated vaccines lies in their ability to stimulate a robust humoral and cell-mediated immune response, which can translate into long-term adaptive protection against subsequent exposure to a virulent wild-type (WT) strain (20, 21, 29). A key step towards live vaccine development lies in understanding *S. iniae* virulence factors. To date, only a handful of these determinants have been characterized for *S. iniae* in the context of fish virulence: phosphoglucomutase (5), capsular polysaccharide (17, 19, 27), Streptolysin S (10, 18), and M-like protein (3, 16).

Mutation of any *S. iniae* virulence gene resulting in significant *in vivo* attenuation warrants preliminary testing of the mutant as a vaccine candidate. Previously, through intraperitoneal (IP) vaccination of HSB, we have found protection levels of 100% against WT *S. iniae* (after 1,400 degree days) with an M-like protein mutant strain (Δ*simA*) (16) and 90-100% protection (after 2,000 degree days) with a phosphoglucomutase-deficient transposon mutant (Δ*pgmA*) (5). Our allelic replacement of the *cpsD* gene in the *S. iniae* capsular polysaccharide biosynthesis operon resulted in a highly attenuated mutant strain (Δ*cpsD*) (17), and preliminary testing showed efficacy of this mutant at higher doses in a similar HSB IP vaccination model (data not shown). Further testing of these vaccine candidates is needed as well as a comparison of their efficacy through multiple delivery methods and against traditional bacterin vaccines.

Here we explore the protective capacity of a formalin-killed *S. iniae* bacterin (WT K288) in parallel with three isogenic live attenuated mutant *S. iniae* strains (Δ*cpsD*, Δ*simA*, and Δ*pgmA*), which are deficient in capsular polysaccharide, M-like protein, and

phosphoglucomutase, respectively. Each vaccine is evaluated through IP injection and bath immersion in juvenile HSB. Survival following a lethal challenge with WT *S. iniae* was used to assess vaccine efficacy after an 800 degree day holding period. These studies are the first to evaluate immersion delivery of live *S. iniae* vaccines and to directly compare efficacy of multiple live *S. iniae* vaccine candidates to a killed bacterin.

Materials and Methods

Bacteria strains, culture, and DNA techniques. *Streptococcus iniae* strain K288 was isolated from the brain of a diseased HSB at the Kent SeaTech aquaculture facility in Mecca, CA (5). The M-like protein (Δ*simA*) (16) and capsular polysaccharide (Δ*cpsD*) (17) allelic replacement mutants and the phosphoglucomutase transposon mutant (Δ*pgmA*) (5) were all generated in the K288 background (Table 5.1). Mutant and WT *S. iniae* strains were grown at 30°C in Todd-Hewitt broth (THB, Hardy Diagnostics) or on agar plates (THA). Enumeration of colony-forming units (CFU) was performed by serially diluting bacteria in PBS and plating on THA. β-hemolytic activity was assessed on sheep blood agar plates (tryptic soy agar with 5% sheep red blood cells). A Colony Fast-Screen Kit (EPICENTRE Biotechnologies) was used to isolate genomic DNA used in PCR identification of *S. iniae* strains isolated from challenged fish.

Formalin-killed bacterin preparation. WT K288 *S. iniae* was grown to early stationary phase (OD_{600} ~0.80). The culture was incubated at room temp for 72 h with 0.07% formaldehyde (V/V). An aliquot of the culture was plated on THA to ensure total killing. Bacteria were centrifuged (3,250 x *g* for 15 min) and resuspended in supernatant to an OD_{600} of 5.0. EMULSIGEN® oil-in-water adjuvant (MVP Laboratories, Inc.) was

added to a final concentration of 20% (V/V) to the bacterial solution. The bacterin was stored at 4°C and used within one week of preparation.

HSB. Juvenile HSB (*Morone chrysops* x *M. saxatilis*, Keo Fish Farms Inc., Keo, AR) were held in a recirculating 1,071-l tank at 20±2°C prior to use in vaccine challenges. All fish were fed daily with 3.5 mm high protein (40%) sinking pellet feed (Nelson's Silver Cup Trout Feed) and were maintained on a 12:12 h (light:dark) light cycle.

IP vaccination. Overnight cultures of *S. iniae* were diluted 1:10 in fresh THB and grown to mid-log phase (OD_{600}=0.40). Cultures were centrifuged at 3,500 x *g* for 7 min, washed once in PBS, recentrifuged, and finally resuspended in an equal volume PBS. Bacterial suspensions were diluted to achieve desired injection doses in a 100 µl injection volume (Table 5.1). Each strain was diluted in PBS and plated on THA to confirm starting inocula. The formalin-killed bacterin as described above was also delivered in a 100 µl volume per fish (~$3x10^{10}$ CFU). Mock vaccinate groups included fish injected with PBS only and a group injected with adjuvant + THB. Groups of 25 juvenile HSB (~15 g) were anesthetized with MS-222 (Western Chemical, Inc.), weighed, and fin clipped (caudal, pectoral, or pelvic) by treatment group. Bacteria were delivered IP using a 1.0 ml syringe and 27.5 gauge needle. Vaccinate groups were held individually in 113-l tanks at 26±1°C with flow-through water and aeration for 2 weeks. Fish were fed sparingly and monitored daily for survival. Brain cultures were taken from mortalities for PCR confirmation of the respective vaccination strain. After the holding period, vaccinates were transferred into 1,071-l recirculating tanks and cohabitated by delivery method. Fish were held for an additional 3 weeks (~800 degree days total) at 20±1°C and fed to satiation daily.

Table 5.1 *S. iniae* vaccine strains and doses

Strain	Description	IP dose	Immersion dose	Ref.
K288	WT fish isolate used for bacterin and live mutants	$3x10^{10}$ CFU	$3x10^{8}$ CFU/ml	(5)
Δ*simA*	M-like protein (SiM) allelic replacement mutant	$3x10^{6}$ CFU	$3x10^{7}$ CFU/ml	(16)
Δ*cpsD*	capsular polysaccharide allelic replacement mutant	$3x10^{7}$ CFU	$3x10^{7}$ CFU/ml	(17)
Δ*pgmA*	phosphoglucomutase transposon insertion mutant	$3x10^{7}$ CFU	$3x10^{7}$ CFU/ml	(5)

Immersion vaccination. A 1.0 l culture of each *S. iniae* strain (mid-log phase) was added to 9.0 l of dechlorinated system water in an aerated, 5.0-gallon plastic bucket (~3.0 x 10^7 CFU/ml final concentration) (Table 5.1). The K288 bacterin was diluted 1:80 in system water (~3x10^8 CFU/ml) for HSB immersion vaccinations. A 10% THB solution served as a mock immersion vaccination control. Fish were anesthetized, fin clipped, and weighed prior to immersion in their respective solutions for a period of 90 minutes. Immersion vaccinated fish were maintained as described for the IP vaccinate groups.

WT *S. iniae* challenge. Vaccinates were removed from the large holding tanks and sorted by treatment group back into the smaller 113-l challenge tanks. Over a 2 day period tanks were warmed up to the challenge temperature of 26°C. Fish were anesthetized and challenged IP with 1 x 10^6 CFU of WT K288. Fish were fed lightly and monitored for survival for 2 weeks post challenge. Bacteria recovered from all mortalities were confirmed to be WT *S. iniae* through culture and PCR analysis. All HSB vaccine trials were carried out in an AAALAC certified facility following IACUC approved protocols.

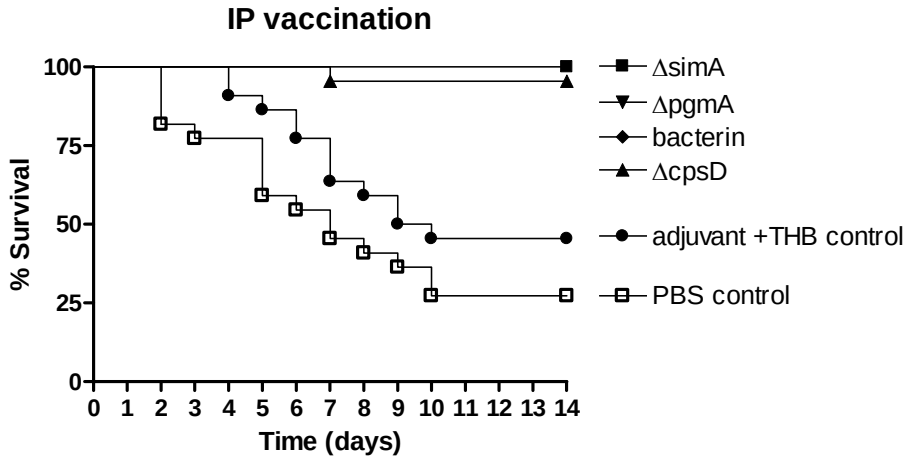
Statistical analysis. Survival data were analyzed using a Logrank test included in the GraphPad Prism software suite (GraphPad Software, Inc.). *P* values less than 0.05 were considered statistically significant. Relative percent survival (RPS) was calculated by the following equation: [1-((vaccinate mortality)/(control mortality))]*100.

Results

Survival post vaccination. Both IP and bath immersion vaccination of HSB using the three attenuated *S. iniae* mutants and the killed bacterin were well tolerated by the fish (Table 5.2). As expected, no post vaccination mortalities occurred in any of the mock vaccination control groups or in the killed bacterin group. Consistent with previous HSB IP virulence studies for the Δ*pgmA* (5) and Δ*cpsD* (17) mutants, IP vaccination with 10^7 CFU of each of these strains generated no mortalities. These two mutants were also completely attenuated when delivered through bath immersion. The Δ*simA* mutant was the only vaccine candidate to generate vaccination-related mortalities in both groups (16% IP, 12% immersion). *S. iniae* recovered from the brains of morts that were vaccinated with the Δ*simA* mutant were positively identified through PCR analysis.

Survival post WT challenge. Fish were held for 5 weeks post vaccination (800 degree days), a sufficient holding period to ensure that any protection against WT *S. iniae* challenge (Fig. 5.1) was a result of adaptive immunity rather than prolonged upregulation of the innate immune system. Following IP challenge of all vaccinate fish with $1x10^6$ CFU of WT *S. iniae*, 27% survival was seen in the THB and PBS mock vaccination groups (Table 5.2). Surprisingly, the THB + adjuvant IP control group had increased survival (45%) compared to the corresponding PBS control group, though the survival curves were not significantly different (P=0.13). No mortalities were observed in fish that had been vaccinated IP with Δ*simA*, Δ*pgmA*, or the killed bacterin. One fish died (5% mortality) in the Δ*cpsD* IP vaccinate group following WT challenge. With the

A



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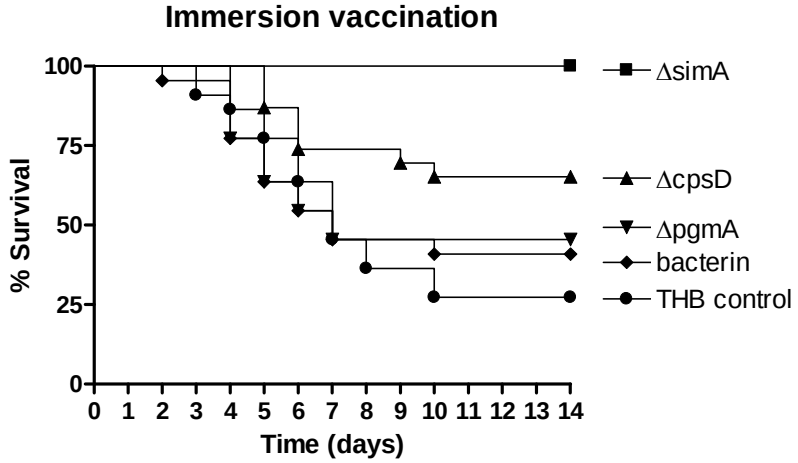

Figure 5.1 Survival of fish vaccinated against *S. iniae*. HSB vaccinated through **(A)** IP injection or **(B)** bath immersion were challenged with a lethal dose of WT *S. iniae* after an 800 degree day holding period. Kaplan-Meier plots show survival of HSB over a 14 day monitoring period post challenge.

Table 5.2 Summary of *S. iniae* vaccine trial data

Delivery Route	Vaccination Group	Vaccination survival (%)	WT challenge survival (%)	Relative % survival
IP Injection	Δ*simA*	84	100	100
	Δ*cpsD*	100	95	93
	Δ*pgmA*	100	100	100
	PBS control	100	27	0
	K288 bacterin	100	100	100
	adjuvant + THB control	100	45	25
Bath immersion	Δ*simA*	88	100	100
	Δ*cpsD*	100	65	52
	Δ*pgmA*	100	45	25
	K288 bacterin	100	40	18
	THB control	100	27	0

exception of the Δ*simA* vaccinate group, where no mortalities were seen, immersion vaccination groups experienced much higher mortality levels than they did through injection: 35% - Δ*cpsD*, 55% - Δ*pgmA*, and 60% - bacterin.

Overall vaccine efficacy. The adaptive immunoprotection conferred by each vaccine was assessed through calculation of relative percent survival (RPS). The Δ*pgmA* and killed bacterin IP challenge groups had perfect safety and protection (100% RPS) profiles during the trial (Table 5.2). However, the Δ*pgmA* and bacterin vaccines were the worst overall immersion candidates with RPS values of 25 and 18%, respectively, not significantly different from the survival of the THB immersion control group (P=0.51, 0.66). The Δ*cpsD* mutant had 93% RPS through IP delivery and this value was not significantly different from Δ*pgmA* and bacterin groups (P=0.37). The Δ*cpsD* immersion vaccinated group had an RPS value of 52% and was significantly better (P=0.01) than the THB immersion control group. The Δ*simA* mutant was the only candidate to generate initial mortalities through IP and immersion vaccination, however it was also the only vaccine to achieve 100% RPS in all surviving fish through both delivery methods.

Discussion

As knowledge of *S. iniae* virulence increases and a growing number of virulence factor-deficient mutants are available, the vaccine potential of the most attenuated of these mutants can be assessed. We evaluated three such *S. iniae* mutants lacking capsular polysaccharide (Δ*cpsD*), M-like protein (Δ*simA*), and phosphoglucomutase (Δ*pgmA*). The efficacy of each live mutant was compared through IP injection and bath immersion in parallel with an isogenic, adjuvanted, formalin-killed bacterin. Both live and killed

vaccine candidates generated 93-100% RPS values when delivered by injection. However through immersion delivery, only the Δ*simA* live mutant provided a high level of protection (100% RPS), demonstrating for the first time the superior efficacy of live *S. iniae* vaccines in this more commercially viable delivery method. This work also highlights some of the challenges faced with live vaccines, such as vaccination safety and the balance between attenuation, efficacy, and the conservation of immunodominant epitopes.

Despite its high protective capacity in both delivery models, the Δ*simA* mutant was the only one of the three live strains tested that lacked complete attenuation. Increased mortality of the Δ*simA* mutant in this IP vaccination study compared with levels seen in previous vaccination experiments using the same dose (16), may be in part be attributed to a higher vaccination/challenge temperatures here than in our previous study (26 vs 24°C), and increase in temperature correlates positively with *S. iniae* virulence (13). Vaccination at cooler temperatures may help increase the safety of lesser attenuated mutants and should be investigated. Also, the fish used in these studies were not from a defined genetic line and can vary from batch to batch in their physical and immunological characteristics. Regardless of any parameters altered, the Δ*simA* mutant will require further mutagenesis-based attenuation. Because the Δ*simA* mutant retained WT-like invasive abilities in the immersion model it suggests that the role of SiM in adherence and invasion of epithelial cells (16) may be less important than the role of this protein in binding host fibrinogen in the bloodstream (3). Because the Δ*simA* mutant was able to provide complete immune protection against WT challenge we speculate that the

SiM protein itself is not an essential antigenic epitope involved in the adaptive immune response to *S. iniae*.

One critical consideration when developing a live vaccine is weighing attenuation versus the respective immunogenicity of the virulence factor that has been removed. The importance of *S. iniae* capsule as an immunogenic epitope has been demonstrated in vaccine studies with killed capsule-deficient bacterins (12, 26) and functionally in aquaculture operations where serotypically distinct isolates with non cross-reactive protective surface antigens emerge in fish populations vaccinated against a single serotype (2, 4). While the 93% RPS of the Δ*cpsD* mutant in the IP group was not statistically significant from the 100% RPS values of the other vaccine strains, we have observed greatly reduced protection levels of this mutant in a previous IP challenge at comparable and lower doses over a longer 1,400 degree day window (data not shown). This suggests that capsule may be an important factor in the efficacy of live *S. iniae* vaccines, but perhaps not to the same extent as it is in killed bacterins.

Properties of an ideal live vaccine include: severe attenuation, low cytotoxicity, complete immune clearance, conservation of key antigenic epitopes, and stimulation of long-term adaptive immune protection. Studies of the Δ*pgmA* mutant, through this trial and earlier work using an extended 2,000 degree day trial (5), show that so far this mutant appears to meet all of these key criteria when delivered through injection. Despite a perfect IP safety and efficacy profile, the Δ*pgmA* mutant was a poor immersion vaccine candidate. Mutagenesis of Pgm appears to have significantly reduced the ability of the bacteria to utilize natural water-based transmission routes, or perhaps weakening of the

cell wall has lowered the ability of this mutant to handle osmotic stress and therefore has diminished viability in water-based immersion delivery.

The adjuvanted, formalin-killed bacterin used in this study was prepared according to a protocol used at an aquaculture facility that vaccinates fish using autogenous *S. iniae* isolates and thus serves a relevant comparative vaccine. The bacterin provided complete protection when injected, though this protective capacity required three logs greater CFU than the highest live mutant IP vaccine doses. Additionally, the bacterin preparation contained a leading commercial vaccine adjuvant, which on its own conferred a survival advantage, though not statistically significant, over the PBS control group within the trial period. This suggests that the protection generated by the K288 bacterin was at least partially attributed to nonspecific immune stimulation conferred by the adjuvant and/or THB. Efficacy of the whole cell bacterin may also be in part due to the inclusion of supernatant form the liquid culture that it was grown in. Previous work with whole-cell killed bacterins has demonstrated the importance of small extracellular products in the adaptive immune response (15). Live vaccines offer the advantage that they inherently produce these small immunogenic compounds *in vivo* and in their native form. Despite high levels of protection when delivered IP, the killed bacterin performed poorly in the immersion trial and did not provide statistically significant protection against WT *S. iniae*.

While injection vaccination is precise and reproducible, it is also labor and cost-intensive and is thus restricted in application to only higher value aquaculture species such as salmonids and HSB. Immersion vaccination is more economical and more closely mimics natural infection routes utilized by aquatic pathogens. IP vaccination

with all vaccines provided >93% RPS, however immersion administration of these same vaccines failed to provide high levels of adaptive immune protection, with the exception of the Δ*simA* mutant. Other studies have shown similar trend with superior protection of killed bacterins for closely related streptococcal sp. when delivered through injection versus immersion (8, 11). The ability to effectively deliver a vaccine through immersion may be a key feature of live vaccines that cannot be replicated with killed alternatives and may make them more attractive to commercial users.

Successful passive vaccination experiments suggest that the adaptive immune response to *S. iniae* infection is largely antibody mediated (9, 24). Kinetic studies of the specific IgM response of fish to vaccination with live *S. iniae* strains would be an interesting comparison to the response generated with killed bacterins. Another avenue of investigation with live vaccines should focus on assessing their cross-protective capabilities. Emergence of serologically distinct strains in operations where fish have been vaccinated is a problem with *S. iniae* vaccination efforts (2). Studies have shown that vaccination with a bacterin composed of multiple serotypes provides a superior protective advantage (15), but just how much cross-protection is generated with live vaccines is unknown.

Live bacterial vaccines have demonstrated sufficient efficacy and safety for use in finfish aquaculture. Two live attenuated vaccines have already received FDA approval for use in US aquaculture: AquaVac-ESC® (Intervet/Schering-Plough Animal Health) for prevention of enteric septicemia in catfish caused by *Edwardsiella ictaluri* (14), and Renogen™ (Novartis Animal Health), an *Arthrobacter davidanieli* strain used to prevent bacterial kidney disease in salmonids caused by *Renibacterium salmoninarum* and

piscirickettsiosis caused by *Piscirickettsia salmonis* (23). These successes give hope that eventually a live *S. iniae* vaccine may be approved for use in aquaculture. Assuming a live *S. iniae* strain demonstrated excellent safety and protective capabilities, two regulatory issues would still need to be addressed. The vaccine strain would need to be incapable of reversion back to WT, an issue not applicable to the allelic mutants, but would be of concern in a transposon mutant. The other main criteria of a live mutant going through commercial development would be the absence any antibiotic resistance genes such as those found in the allelic replacement and transposon mutants examined in this study. Both of these concerns are easily resolved through subsequent targeted deletion mutagenesis.

In sum, we have evaluated a panel of three attenuated live *S. iniae* vaccine candidates along side an adjuvanted bacterin using injection and immersion delivery. While high levels of protection were achieved with all vaccines candidates through injection, the Δ*simA* mutant was the only vaccine to provide complete protection through immersion delivery, and may highlight an advantage of invasive, yet attenuated live vaccines over killed bacterin alternatives. Work is underway to further attenuate the Δ*simA* mutant through targeted mutagenesis of additional virulence factors in order to achieve a more desirable safety profile. Future large-scale studies will investigate long-term and multi-species efficacy of the most promising live vaccine candidates. Oral delivery of live attenuated vaccines is also under evaluation because this delivery method, like immersion delivery, utilizes a potential *S. iniae* transmission route and may be well suited for use with live attenuated mutants.

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Chapter VI

Conclusions and perspectives

Introduction

Declining natural fisheries and increased demand for seafood products will continue to fuel the worldwide growth of aquaculture into the foreseeable future. While aquaculture does provide an attractive alternative to wild capture fisheries, there are a few key issues, such as disease, which need to be more adequately addressed in order to reduce environmental impacts and improve profitability of these operations. Suboptimal conditions and stressors (i.e. temperature fluctuations, poor water quality, handling, crowding, etc.) experienced by farm-raised fish make them more prone to opportunistic pathogens such as *Streptococcus iniae* (6, 27, 30, 32). *S. iniae* is a threat not only to fish raised in intensive culture, but also to nearby wild fish populations (10, 14, 38) and to people who handle fresh fish (36). There is a pressing need for novel therapeutic options to treat or prevent *S. iniae* infection. Though antibiotics have demonstrated experimental efficacy against *S. iniae* (11-13, 35), none are officially approved for use against this pathogen in US aquaculture. Vaccination appears to be the most promising therapeutic approach, however the use of killed bacterin vaccines so far has been met with limited success and problems with serotypic reemergence (1, 2).

One of the limiting factors in the development of novel therapeutics for *S. iniae* lies in the lack of understanding of how this pathogen causes disease. Taking advantage of the tools and discoveries made in over a century of research on major human streptococcal pathogens such as group A and B *Streptococcus* (GAS, GBS), our understanding of *S. iniae* pathogenesis has come at a more accelerated pace. Transposon mutagenesis has been a powerful tool for identifying virulence determinants (9, 25, 34) and more recent genomic sequencing efforts have enabled informed targeted mutagenesis

of virulence factor candidates with homology to proven virulence determinants in other pathogenic streptococci (18, 37). At the commencement of the research presented here, only a single gene, phosphoglucomutase, through previous work in our laboratory (9), had been established as an *S. iniae* virulence factor in the context of fish pathogenesis.

The work contained herein describes the mutagenesis and characterization of four *S. iniae* gene homologues to some of the most highly studied virulence factors in GAS and GBS pathogenesis. Streptolysin S (SLS) and capsular polysaccharide biosynthesis gene clusters were identified in *S. iniae* through work by others who sequenced the insertion sites of transposon mutants (16, 22). Our identification of the M-like protein (*simA*) and the C5a peptidase-like protein (*scpI*) genes was done through BLAST analysis of contigs generated from whole genome pyrosequence data (454 Life Sciences) against annotated GAS genomes (18). Supporting phylogenetic classification of *S. iniae* in between GAS and GBS, we also see a similar mixture of virulence factor homologies between these strains as well as some unique properties to *S. iniae*.

Analysis of virulence factor mutants

The *S. iniae* SLS biosynthesis operon (16) is homologous to the SLS operon of GAS (29) and distinct from the β-hemolysin of GBS (31). Generation of the SLS-deficient Δ*sagA* mutant and marked attenuation in HSB led us to hypothesize that reduced cytotoxicity, to brain cells in particular, may be responsible for the reduction in virulence (20). *In vivo* activity of SLS against red blood cells should also be investigated however, as a reduction in hematocrit levels is a common finding in fish with systemic *S. iniae* infections (15, 24). Further research on the role of this toxin in *S. iniae*

meningoencephalitis is needed because the mutant was able to persist even at significant levels (10^6 CFU/g) in the brains of some fish even after it had been cleared from the bloodstream and other major organs (20). In fish held for extended periods (>4 months) post challenge with Δ*sagA*, we observed outbreaks of lethal infection definitively caused by the mutant as water temperatures rose from 14 to 20°C in the holding facility (data not published). Because of these incomplete attenuation issues the Δ*sagA* mutant was not pursued as a vaccine candidate.

S. iniae capsular polysaccharide biosynthesis is accomplished by a 21-gene operon with much higher similarity to the capsule operon of GBS rather than GAS (22). Our studies with the Δ*cpsD* capsule mutant were the first to confirm with precise targeted mutagenesis that this extracellular coating is a critical, if not the most critical, virulence factor that *S. iniae* possesses primarily because of its ability to resist phagocytic clearance (19). We provided preliminary biochemical analysis of monosaccharides that may make up *S. iniae* capsule, but further work is needed to define the secondary and tertiary capsule structure. Investigation of capsule regulatory mechanisms during various stages of disease pathogenesis will also be important, as we have demonstrated that presence of capsule is essential for survival in the bloodstream and resistance to phagocytosis, yet a lack of capsule may be critical to *S. iniae* adherence and invasion of host tissues (19). Work by others has established *sivR/S*-mediated capsule regulation (4, 5), though a number of other putative capsule regulatory genes have been identified through transposon mutagenesis (9, 22, 25, 34) as well as our analysis of genomic pyrosequence data (i.e. serine/threonine phosphatase and kinase, covR/S, etc.). It is worth investigating the nature of serotypic variance and whether Serotype II strains have increased capsule

because of differences in regulation or if any potential differences in capsule composition or abundance may be due to genetic variation within the capsule operon itself.

Whole genome pyrosequencing (23) identified a Mga-like locus in *S. iniae* containing an M-like protein (*simA*) and adjacent *mga*-like regulatory gene (*mgx*) which is likely to be a significant regulatory factor in *S. iniae* virulence (3, 18). The presence of an M family gene next to a *mga*-like regulatory gene is similar to GAS, but the divergent transcription of these *S. iniae* homologues is a unique feature that has only been described so far in the livestock pathogen *S. uberis* (26). The region upstream of *mgx* is also an area of interest because of what appears to be a distant gene duplication or multiple integration event (18). The naturally occurring deletion mutation and subsequent generation of a second full length *mgx* gene in strain 02161A warrants investigation to determine if there is any functionality to this ORF. Further sequencing efforts will be needed to confirm the high degree of conservation seen so far in *S. iniae sim* genes. Additional studies will help to elucidate functional differences between the two *sim* alleles (*simA, B*) as well as the two truncated *simA* ORFs that we identified in the attenuated 02161A strain (3, 18).

Our discovery of the *S. iniae* C5a peptidase homologue revealed that this gene is not located within the *mga*-like *mgx* locus in *S. iniae* as it is in GAS (18). The chromosomal positioning of *scpI* is most similar to group C and G streptococci (which also possess *mga*-like loci) and to GBS (does not possess M family proteins or Mga regulators). We were surprised that the Δ*scpI* allelic replacement mutant was not attenuated in HSB or zebrafish infection models (18) or in its ability to bind to fish epithelial cells or human fibronectin (data not published). Before excluding this protein

completely from a role in virulence further *in vitro* analyses are needed. ScpI does contain the Asp-His-Ser catalytic triad amino acid residues found in the active sites of GAS and GBS C5a peptidase homologues. C5a cleavage assays will reveal any proteolytic capacity of this protein, and if such activity is observed, transwell chemotaxis assays employing fish neutrophils could be used to show functionality of *ScpI ex vivo*.

Future work will continue the analysis of putative virulence associated genes identified in our pyrosequence data (i.e. hyaluronidase, Fc receptors, serine threonine phosphatase/kinase, covR/S, streptodornase, CAMP factor, C3 proteinase, etc.). Already one additional gene identified through this sequencing effort, *cepI* (cell envelope proteinase, IL-8 protease), has been characterized through work in our lab using a mouse infection model (37) and future studies will determine if this gene plays a role in virulence towards fish.

With the upcoming release of the first official whole genome sequencing project (www.hgsc.bcm.tmc.edu) comparisons between this human isolate (9117) and our virulent fish isolate (K288) can be made. Additional whole genome sequencing efforts will help elucidate the basis for serotypic variation (2) and strain-associated virulence trends (8). Access to full genome sequence will also facilitate analysis of *S. iniae* virulence regulators through transcriptional analysis of virulence genes using real time PCR and microarrays. Transcriptional analysis of virulence genes during various pathogenic stages and niches (i.e. in the presence of serum, within phagocytes, at various temperatures, etc.) will also help provide a greater understanding of *S. iniae* virulence. Finally, genomic comparisons will also help better define *S. iniae* phylogenetically among other streptococci.

Live attenuated vaccine development

The use of targeted mutagenesis is a powerful tool in the identification and characterization of *S. iniae* virulence factors and this technique has the added benefit that the most highly attenuated strains may have utility as live vaccines. Of the four mutant strains generated in this work, the Δ*cpsD* capsule and Δ*simA* M-like protein mutants demonstrated the highest vaccine potential (21). While the complete attenuation of the Δ*cpsD* mutant made it the more attractive candidate with respect to safety, the Δ*simA* mutant provided superior immune protection, especially through immersion vaccination. Future work with this mutant will focus on further attenuation through allelic replacement of one or more additional virulence factors. The subsequent deletion of *sagA* form the Δ*simA* mutant would be a logical starting point because we found that SLS contributes significantly to *S. iniae* virulence, though is not involved in adherence or invasion (20), and GAS SLS has been shown to be non-immunogenic -- all of which are key properties when selecting a virulence factor to inactivate in the development of a live vaccine for aquaculture.

Analysis is underway on *S. iniae* IgM antibody titers measured during the live vaccine trials and these data will help to characterize the adaptive immune response to live vaccination and elucidate differences in the IgM response to live versus killed vaccines. Future IgM-based analyses may help address whether live vaccines are capable of offering greater cross-protection against multiple serotypes than has been shown with killed bacterins, or if live vaccines may need to be developed for multiple serotypes and mixed together to provide comprehensive protection.

Oral delivery is another area of great interest for vaccine development in aquaculture. This strategy has the potential to be the ultimate low cost and low stress mechanism for vaccine delivery to fish. Even if initial oral vaccination had a limited protective window, "booster" administrations of the vaccine could easily be delivered throughout the grow-out cycle to provide complete protection. While killed *S. iniae* bacterin vaccines have shown limited efficacy in immersion (17) and oral (33) delivery trials, live vaccines may offer a superior alternative in these more economical delivery methods which more closely mimic natural transmission routes of *S. iniae* (7, 28). Work was attempted to establish infection in HSB through a variety of oral delivery methods with WT *S. iniae*, but was met with limited success (data not published). Oral delivery studies with attenuated mutants and the use of immunoassays to measure anti-*S. iniae* IgM will be required to see if live vaccination can generate protective immunity through this method in HSB, even if similar delivery of WT *S. iniae* fail to generate infection.

Final thoughts

In a relatively short amount of time our understanding of *S. iniae* pathogenesis has increased significantly due to the characterization of four putative virulence factors: streptolysin S, capsular polysaccharide, M-like protein, and C5a peptidase. Knowledge of each of these virulence determinants has the potential to inform development of novel therapeutics in the form of live or subunit vaccines or by providing targets for development of small molecules or peptides with anti-virulence factor activities. Vaccination has the greatest therapeutic potential for *S. iniae* and the ability of the Δ*simA* M-like protein mutant to provide complete immune protection through injection and

immersion delivery is very encouraging. As the aquaculture industry continues to expand so will the prevalence of opportunistic pathogens like *S. iniae*, however we are now in a better position to treat or prevent outbreaks because of the knowledge gained from studying the factors which allow this aquatic bacterium to cause disease.

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