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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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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-

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).

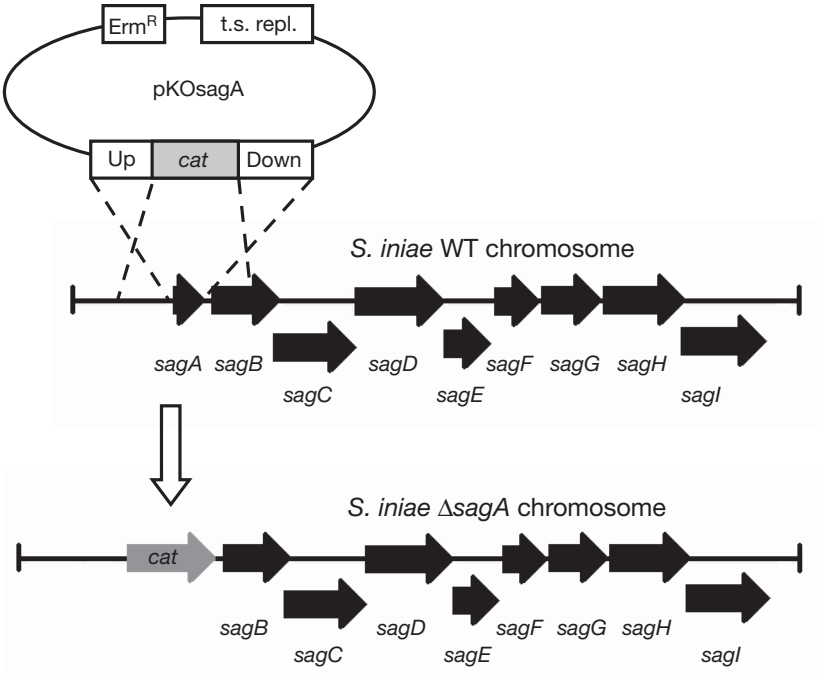

Fig. 1. Allelic exchange mutagenesis scheme for replacement of the *sagA* gene of the steptolysin S (SLS) operon with *cat.* The pKOsagA plasmid contains the fusion PCR composed of ~400 bp *sagA*-flanking regions upstream (Up) and downstream (Down) nesting the *cat* gene in between. The plasmid also 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

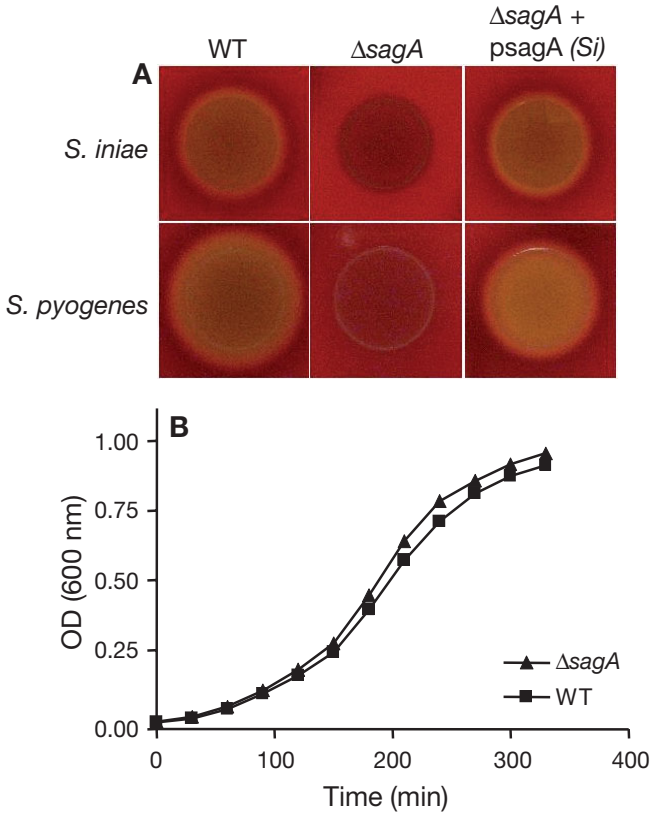

Fig. 2. Functional validation of the Δ*sagA* mutant. (A) Deletion of *Streptococcus iniae sagA* (Δ*sagA*) results in loss of hemolytic activity. The SLS⁻ mutant phenotype, in both *S. iniae* and *S. pyogenes*, can be rescued by complementation using *S. iniae sagA* on a plasmid, psagA. (B) Growth rate 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

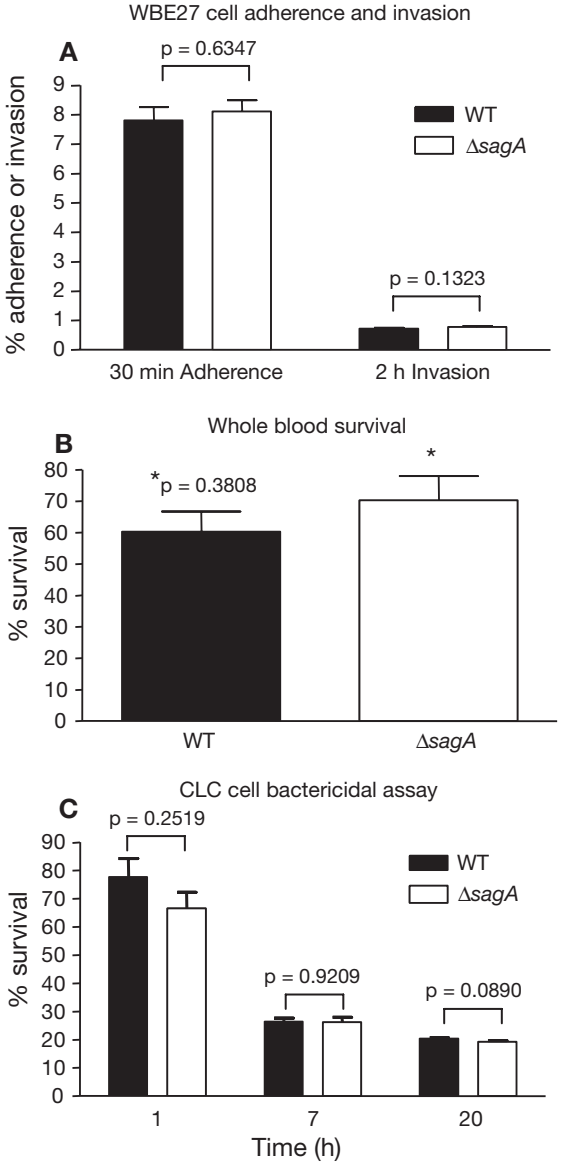

Fig. 3. Lack of SLS contribution to certain *Streptococcus iniae* virulence phenotypes *in vitro*. (A) Adherence and invasion characteristics of wild-type (WT) *S. iniae* and the Δ*sagA* (SLS⁻) mutant for fish epithelial cell line WBE27. (B) Survival of WT and the Δ*sagA* mutant in whole hybrid striped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

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.

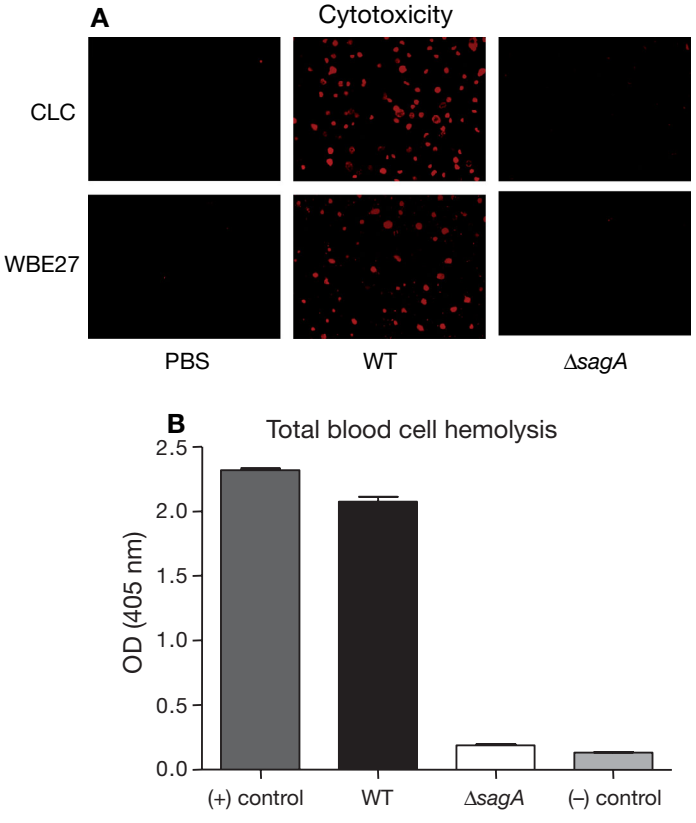

Fig. 4. Assessment of cytotoxic effects of SLS on fish epithelial, macrophage, and blood cells. (A) Cytotoxicity of wild-type (WT) *Streptococcus iniae* and the Δ*sagA* mutant toward white bass epithelial (WBE27) and carp leukocyte culture (CLC) cells following a 5 h incubation at a multiplicity of infection (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

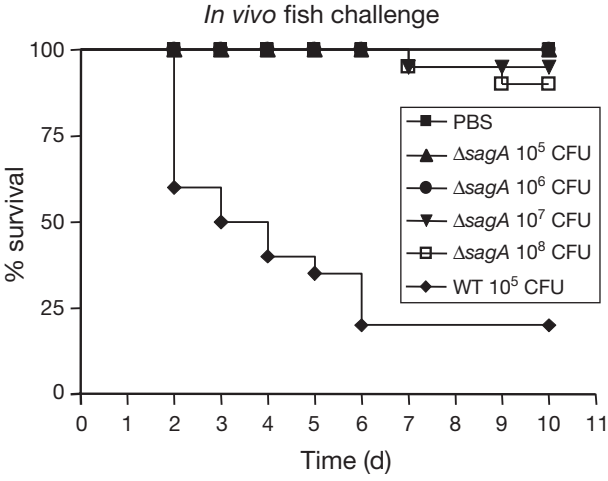

Fig. 5. Kaplan-Meier survival plot of hybrid striped bass (HSB) infected with wild-type (WT) *Streptococcus iniae* (3×10^5 CFU), the Δ*sagA* (SLS$^-$) mutant (3×10^5, 10^6, 10^7, or 10^8 CFU), or phosphate-buffered saline (PBS)

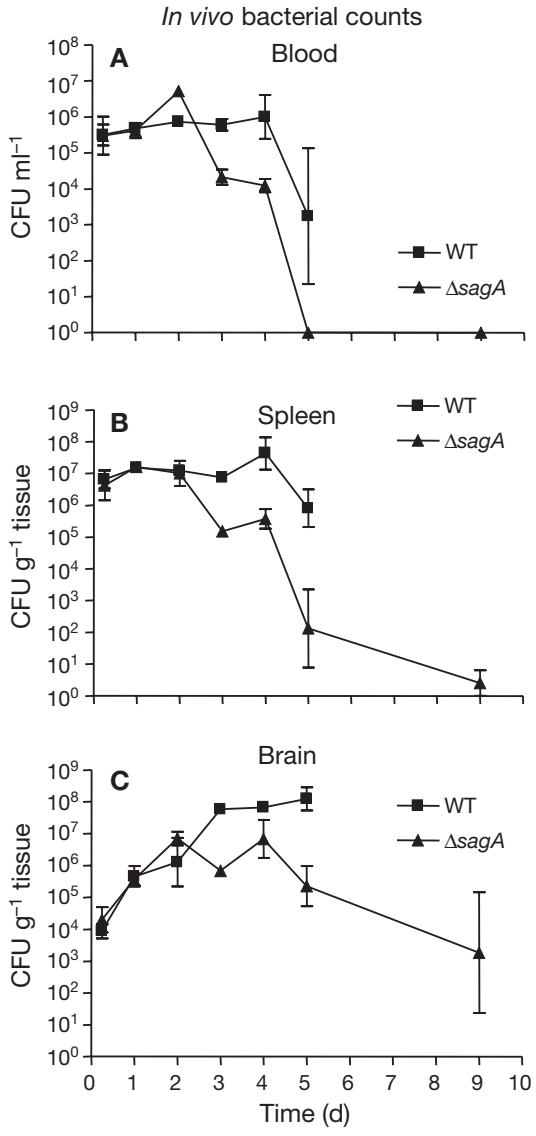

Fig. 6. Characterization of the systemic dissemination of wild-type (WT) *Streptococcus iniae* and the isogenic Δ*sagA* mutant in hybrid striped bass (HSB). CFUs are averages of levels detected in the (A) blood, (B) spleen, and (C) brain of 4 fish per group at 0.25, 1, 2, 3, 4, 5, and 9 d post challenge

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

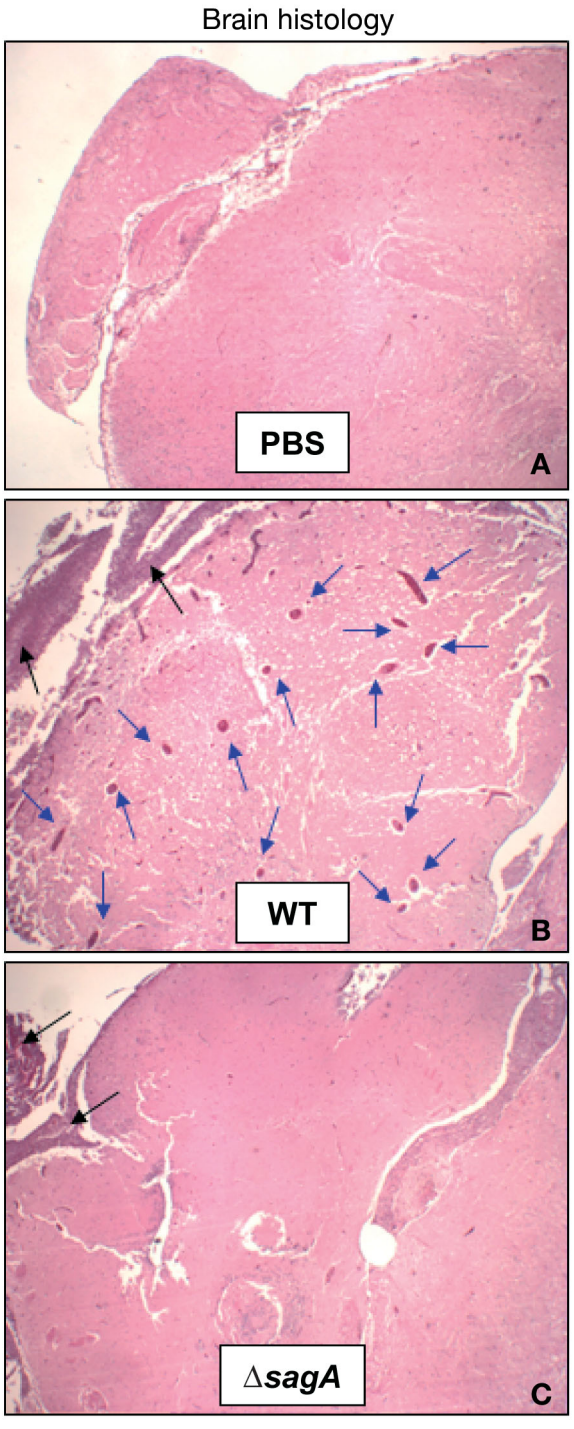

Fig. 7. Histological analysis of brain tissue of hybrid striped bass (HSB). Sagittal brain sections were collected 4 d post challenge from fish injected with (A) phosphate-buffered saline (PBS), (B) 3×10^5 wild-type (WT) *Streptococcus iniae* or (C) the Δ*sagA* mutant. Sample sections were stained with 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)

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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