**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* (*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 AsmA 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 meningococcal meningitis 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 [3,6]; 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
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 125.3 kDa. BLAST (blastn) 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 (ScpA, 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 LPXTG 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 (blastn, 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 (blastn) to the Mgx protein of S. iniae QMA0076 (42% identity, 58% positive) [29], the DmgB Mga-like protein of GCS
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-
tase gene (pgmA) which has been implicated in S. iniae fish virulence [9]. Upstream of scpI lies a 957 bp putative 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 adjacent 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 ΔsimA or ΔscpI 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 ΔsimA mutant did enter stationary phase at a slightly higher optical density than either the WT K288 or the ΔscpI mutant (Fig. 5D) and the ΔscpI mutant had a slightly increased frequency of multimeric coci 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 ΔsimA 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 ΔsimA mutant was required to generate comparable mortality to WT K288 (data not shown). Similar to the K288 ΔsimA mutant, S. iniae WT 02161A strain (with a frameshift mutation truncating the simA

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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      | internal organs |          |
| 94290    | LSU        | Louisiana  | tilapia  | brain         | [9]       |
| 95006    | LSU        | Louisiana  | tilapia  | abscess       | [5]       |
| 94449    | LSU        | Louisiana  | tilapia  | abscess       |           |
| 9117     | UT         | Ontario    | human    | blood         | [3]       |
| 9066     | UT         | Ontario    | fish (sp. | 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.

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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 pKOsclp) containing ~1,000 bp flanking regions upstream (Up) and downstream (Down) nesting the cat gene in between. The plasmid also contains Erm resistance (ErmR) 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).

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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 DsimA 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 DscpI 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 DsimA mutant to three AMPs: Bacillus-derived polymyxin B, HSB derived-moronecidin, and murine-derived CRAMP. Both WT and DsimA 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% DsimA killing by 60 μM polymyxin B in 120 min; 99.21±0.06% WT vs. 98.74±0.14% DsimA killing by 1.5 μM moronecidin in 15 min; 99.98±0.05% WT vs. 99.92±0.47% killing by 10 μM CRAMP in 30 min.

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/deletion (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.

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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.

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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 *vivo* [45]. Compared to the WT parent strain K288, the *S. iniae* ΔsimA 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 ΔsimA 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 ΔsimA 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 ΔsimA 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).
The attenuated \( \Delta simA \) 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 \( \Delta simA \) 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 \( \Delta simA \) mutant (3\( \times \)10^4 and 3\( \times \)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 (LD96, 5\( \times \)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...
that may play out over time in \( \text{O}2161\text{A} \) to generate a novel satory mutations that bring the gene back into frame are proposed HS\( \text{B} \) model. In GAS, frameshift mutations followed by compen-

unknown function, and note that this strain is attenuated in the \( \text{O}2161\text{A} \) that splits the coding region into two smaller ORFs of de
determinate.

the degree of investigation. Additional sequencing efforts are needed to gauge allele types for M-protein in GAS [63]. The functional sequences [29]. These findings contrast the extreme variation in strain QMA0141 as part of a similar comparative analysis of allele. The only other documented SiM proteins. Sequencing a panel of 11 diverse insertional frameshift mutation of the \( \text{S. iniae} \) gene represent an alternative

whether the mutations leading to a disrupted \( \text{simA} \) gene and the creation of a second putative \( \text{mgx-like} \) gene sequence divergence and to investigate the regulation of these genes and their specific C5a peptidase-like protein does not. Future research will

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 \( \Delta \text{simA mutant} \).

A primary 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 \( \Delta \text{mgx2} \) 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 \( \text{S. iniae} \) 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 \( \text{S. iniae} \) [9]. Though limited mortality was observed in our vaccinations with the \( \Delta \text{simA mutant} \), 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 \( \text{S. iniae} \) 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 \( \text{S. iniae} \) 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 \( \Delta \text{simA mutant} \) as a live attenuated vaccine in aquaculture.

**Materials and Methods**

**Bacteria strains, culture, transformation, and DNA techniques**

The WT virulent \( \text{S. iniae} \) 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

\[ \text{Table 2. Immune protection conferred by the } \Delta \text{simA mutant in HSB.} \]

| Vaccination group | % Survival: \( \Delta \text{simA} \) vaccination | % Survival: WT K288 challenge |
|-------------------|---------------------------------|---------------------------------|
| 3 x 10⁸ CFU       | 92                              | 100                             |
| 3 x 10⁹ CFU       | 92                              | 100                             |
| PBS control       | 100                             | 4                               |

MgA-likelike genes. The functionality of \( \text{mgx2} \) in strain 02161A, and whether the mutations leading to a disrupted \( \text{simA} \) gene and the creation of a second putative \( \text{mgx-like} \) gene represent an alternative virulence strategy in this strain, are interesting areas for investigation. Sequence upstream of \( \text{simA} \) with strong similarity to the Mga transcriptional regulatory binding domains of GAS M proteins suggests that Mgx regulation of \( \text{simA} \) expression is likely to occur in \( \text{S. iniae} \). 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 endopeptidolytic activity against the complement system polymorphonuclear leukocyte chemotaxin C5\( \alpha \) [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 \( \text{S. iniae} \) \( \text{scpI} \) 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, \( \text{S. iniae} \) may possess other gene encoded determinants with functional redundancy to \( \text{ScpI} \), 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 \( \text{S. iniae} \) isolates generated only one significant sequence variation. We report an insertion/deletion mutation of the \( \text{simA} \) gene in \( \text{S. iniae} \) 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 \( \text{sim} \) allele. The only other documented \( \text{sim} \) allele, \( \text{simB} \), was found in strain QMA0141 as part of a similar comparative analysis of \( \text{sim} \) sequences [29]. These findings contrast the extreme variation in allele types for M-protein in GAS [63]. The functional implications of this conservation among \( \text{sim} \) alleles warrant further investigation. Additional sequencing efforts are needed to gauge the degree of \( \text{S. iniae} \) M-like protein sequence divergence and to determine if this surface protein may contribute as a serotyping determine.
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' - and 3' -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 (Cat and Erm) 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, ΔsimA and ΔscpI.

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/phrapconsed.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 ΔsimA and ΔscpI allelic mutants.

| Number | Primer name | Sequence (5' – 3') |
|--------|-------------|--------------------|
| 1      | catF        | atggagaaaaatacctgtgatccaccc |
| 2      | catR        | ttaacgccccgccgctgcacacttgcagta |
| 3      | simA–1063F  | agggacagaaaccttcagacag |
| 4      | simA–1015F  | agtcctggtcataacttgcag |
| 5      | simA–22cat22R | ggtatatccagtgatttttttctccatgtttagggttctccttattttc |
| 6      | cat635simA+25F | gcgatgagtggcagggcggggcgtaagctttcccttgcaaccttttcatag |
| 7      | simA+1016R  | aagtcacagggctgctgcagcttg |
| 8      | simA+1152R  | agattcgccggaactcgcttg |
| 9      | scpI–859F   | aqacgatacatcaggtaaggtaag |
| 10     | scpI–821F   | ttagcactcttctcgacgctc |
| 11     | scpI–18cat27R | ggtatatccagtgatttttttctccatgtttagggttctccttattttc |
| 12     | cat635scpI+19F | gcgagttgcgagggcggggcgtataacttttcactaaagtttccacatag |
| 13     | scpI+1044R  | acaaaaattgtgtagagttatg |
| 14     | scpI+1176R  | ttcatttggaaccttacttg |

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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 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 EU699238 and EU714186, respectively. Sequences for scpI and flanking genes in strain K288 were deposited under the accession number EU699239.

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. SimA/P version 3.0 algorithm was used to screen the protein 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 Phylib 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 Ca²⁺ or Mg²⁺) 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 x 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 x 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 x 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 ΔsimA and ΔscpI mutants was assayed in juvenile (~15 g) HSB (Morum chrysops×Morum savastis) as previously described [7]. Groups of 10 fish per treatment group were injected intraperitoneally (IP) or intramuscularly (IM) in the dorsal muscle with 3 x 10⁵ 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 x 10⁴ CFU of ΔsimA, ΔscpI, or WT K288 as previously described [40]. Mid-log phase bacteria were diluted in PBS and injected in 10-µl volumes using a 0.5 cc syringe with a 29-gauge needle. Fish were maintained at 28 °C in recirculated, 104 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 H₂O 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 x 10⁵ 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 x 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 (bacteriaccells). Following centrifugation at 350 x g for 10 min, the plate was incubated for 60 min at 28 °C with 5% CO₂. 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 ΔsimA 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<sup>4</sup> or 3 × 10<sup>5</sup> CFU of the ΔsimA mutant or with PBS alone. Fish were held at 24°C for 2 weeks in 113-l aerated, flow-through tanks. Fish were harvested 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 for a period of 2 days. Each group was then challenged with a 100-μl IP injection of 5 × 10<sup>5</sup> 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 SmA and M family proteins with highest similarity. Strain abbreviations: Sln-S. iniae, SPy-S. pyogenes, SUB-S. uberis, SEq-S. equi, and SDy-S. dysgalactiae

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**Figure S2** Amino acid alignment of ScpI with GAS and GBS C5a peptides. ScpI shows high sequence similarity to the closest C5a peptide homologues from GAS (ScpA, SPy Manfredo M5 strain) and GBS (Cscp B, SAg A909 strain). ScpI possesses the conserved LPXTG 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.

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

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